ENDOCRINOLOGY

VOLUME 47 DECEMBER, 1950 NUMBER 6

# ALLOXAN ADMINISTRATION IN THE GUINEA PIG

## A STUDY OF THE REGENERATIVE PHASE IN THE ISLANDS OF LANGERHANS[1]

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THE guinea pig appears to differ from other laboratory animals in its reaction to the drug alloxan. In such animals as the rabbit, rat, dog, monkey, cat and mouse, administration of alloxan results in necrosis of the B cells of the islands of Langerhans and the consequent diabetic state. Although degenerative lesions in the islet cells of the guinea pig have been reported following alloxan administration, permanent diabetes has not been produced in this species (Goldner and Gomori, 1944; Saviano and De Franciscis, 1947; West and Highet, 1948; Griffiths, 1948; Charalampous and Hegsted, 1949; Collins-Williams *et al.*, 1950; Johnson, 1950).

During the course of a recent study (Johnson, 1950), it was noted that although the islet tissue, and in particular the B cell component, undergoes severe degenerative changes within a few hours after alloxan administration, there is complete restoration of the islet tissue by the fourth day. This recovery coincides in time with the return of the blood sugar to the normal level. The islet tissue at four days and also at six months after alloxan was found to consist of heavily granulated A and B cells in islets of normal size and distribution. In addition, there were many small islets composed almost exclusively of B cells. No vestige of necrotic B cells remained at four days. It seemed of interest, therefore, to study further the regenerative phase in the islet tissue and also to determine, if possible, the source of the new B cells. Do the few undamaged B cells which always seem to remain, regardless of the dosage of alloxan, proliferate, or is there a differentiation of B cells from duct or acinar cells?

Received for publication June 8, 1950.

[1] Aided by a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation.

## MATERIALS AND METHODS

Careful histological observations were made of the islet tissue and in particular of the position and appearance of the B cells at intervals after alloxan administration. In addition, in one series of experiments, colchicine was used to arrest mitotic divisions in metaphase. This afforded a means of determining the degree of cellular proliferation in the various types of cells of the pancreas after alloxan administration.

Fasting guinea pigs weighing between 422 and 1050 grams were given intravenous injections of alloxan[2] (150 or 200 mg. per kilo) and sacrificed 6, 12, 18, 24, 48 and 77 hours later. Colchicine in freshly prepared 0.5% aqueous solution (0.3 mg. per kilo) was injected subcutaneously 9 hours prior to sacrificing the animals. Control animals were given the colchicine injection only and sacrificed 9 hours later. Small pieces of the tail of the pancreas were fixed in Bouin's fluid, sectioned at 4$\mu$ and stained with chrome hematoxylin and phloxin (Gomori, 1941).

## RESULTS

The degenerative changes in the islet tissue produced by alloxan have been dealt with in detail elsewhere (Johnson, 1950). Necrosis of the B cells is apparent at 2 hours and is progressive up to about 24 hours. By the end of the second day (48 hours after the alloxan injection) nearly all of the necrotic B cells have disappeared. There remain occasional pyknotic nuclei and a few areas of homogeneous gray-blue staining cytoplasmic remnants. The islets are otherwise filled with A and B cells and appear quite normal at a glance. However, many of the B cells are unusually large and lightly granulated, particularly at the periphery of the islets, and there are more than the usual number of points of possible acino-insular transformation, regions where the islet cells and acinar cells are in direct relation without any intervening connective tissue. Frequently in these regions the acinar cells are pale-staining, without the usual amount of chromatophilic material or zymogen granules. In some instances, groups of B cells within an islet but near the periphery are arranged in the form of an acinus. The number of very small islets appears to be increased. These islets are made up almost exclusively of B cells, and often appear either as buds from the small ducts or as transformed acini (Figure 2). Those which are in the form of acini often show distinct cell boundaries and a central lumen, which is not the case in the untreated guinea pig. As a consequence of the large number of B cells in the small islets, the proportion of B to A cells appears to be greater than in the normal. This impression is substantiated by actual cell counts made four or more days after alloxan (Johnson, 1950). On the fourth, fifth and twelfth days the islet tissue appears normal except that the outlines of some of the islets are still indistinct due to the absence of any connective tissue partition between islet and acinar cells (Figure 1).

Having shown that there is a rapid replacement of the B cells in the

[2] Alloxan (monohydrate), Eastman Kodak Company, Rochester, N. Y.

pancreas of the guinea pig treated with alloxan, there remained the problem of determining the source of these replacement cells. Three control and 14 experimental animals were injected with colchicine. The experimental animals were also given 150 or 200 mg. alloxan per kilo intravenously and sacrificed 6 hours (1 animal), 12 hours (3 animals), 18 hours (3), 24 hours (3), 48 hours (2) and 77 hours (2) after alloxan administration.

In none of the experimental animals was there an increase in mitoses in either the islets or the acinar tissue in the above periods following alloxan administration. For example, the average number of mitotic figures found in sections from 3 controls was 42 in acini and 4 in islets; and in 14 experimentals, 9 in acini and 2 in islets. Several of the alloxan-treated animals showed no cell divisions in the islets, and a few showed no dividing acinar cells.

It has been stated earlier that there appeared to be many points of acino-insular transformation in the animals which lived 2 days or more after alloxan administration. Points of acino-insular transformation are not absent in the normal guinea pig, but are relatively few in number. With careful microscopic study, what appeared to be actual transition cells were found in the alloxan-treated animals. The possibility of mistaking a B cell overlying an acinar cell for a true transition cell was eliminated by examination of adjacent serial sections.

The transition cells may be of four different types: dedifferentiated acinar cells containing no zymogen granules, very little chromatophilic material and a few fine, scattered B granules (Figure 3); cells containing a small clump of zymogen granules apically, little or no chromatophilic material, and with B granules basally (Figure 4); cells without zymogen granules, but containing both chromatophilic material and small clumps of B granules (Figure 5); and centroacinar cells with accumulating B granules. The usual sequence, judging from frequency of occurrence, appears to be the loss of zymogen granules in individual acinar cells bordering on an islet, followed by loss of chromatophilic material (dedifferentiation) and gradual accumulation of B granules in these cells (redifferentiation). Almost invariably the transition cells are found adjacent to a capillary. Small ducts also appear to contribute to the formation of new islets as evidenced by groups of B cells which appear as buds at the termination of some of the smallest ducts. Formation of new B cells was most pronounced in the animals sacrificed 18, 24 and 48 hours after alloxan administration.

## DISCUSSION

The replenishment of the B cells as judged by the colchicine technique is not due to an increase in the mitotic divisions of the few remaining B cells, for the number of mitoses in them is not greater than normal. Mitotic

figures were observed occasionally in the islets of the normal guinea pigs not treated with colchicine, and also in islets with degenerating B cells in the alloxan-treated animals. Proliferation by division of pre-existing islet cells is known to take place under various conditions which result in an increased demand upon the islet tissue (Florentin and Watrin, 1931; Woerner 1938; Richardson and Young, 1938; Vazquez-Lopez, 1940). Mitoses have also been found in the islets of the rabbit and rat following repeated small doses of alloxan (Bailey, Bailey and Leech, 1944; Hughes, 1947) and at 72 hours after a diabetogenic dose in the rat (Di Pietro, 1945). The small number of mitotic divisions in both acinar and islet tissue in the alloxan-treated guinea pig as compared with the normal may be due to the general toxic effects of the drug. Many of the animals showed severe renal and hepatic lesions as well as hemolysis and anemia.

The replenishment of the islet tissue in the alloxan-treated guinea pig so far as can be judged from observation of fixed material appears to be due chiefly to two processes. One of these is the budding of small ducts and the transformation of the cells of the buds into B cells. Occasionally in

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PLATE 1

EXPLANATION OF FIGURES

All sections cut at 4$\mu$ and stained with chrome hematoxylin-phloxin; B granules stain blue; A granules pink; zymogen granules red.

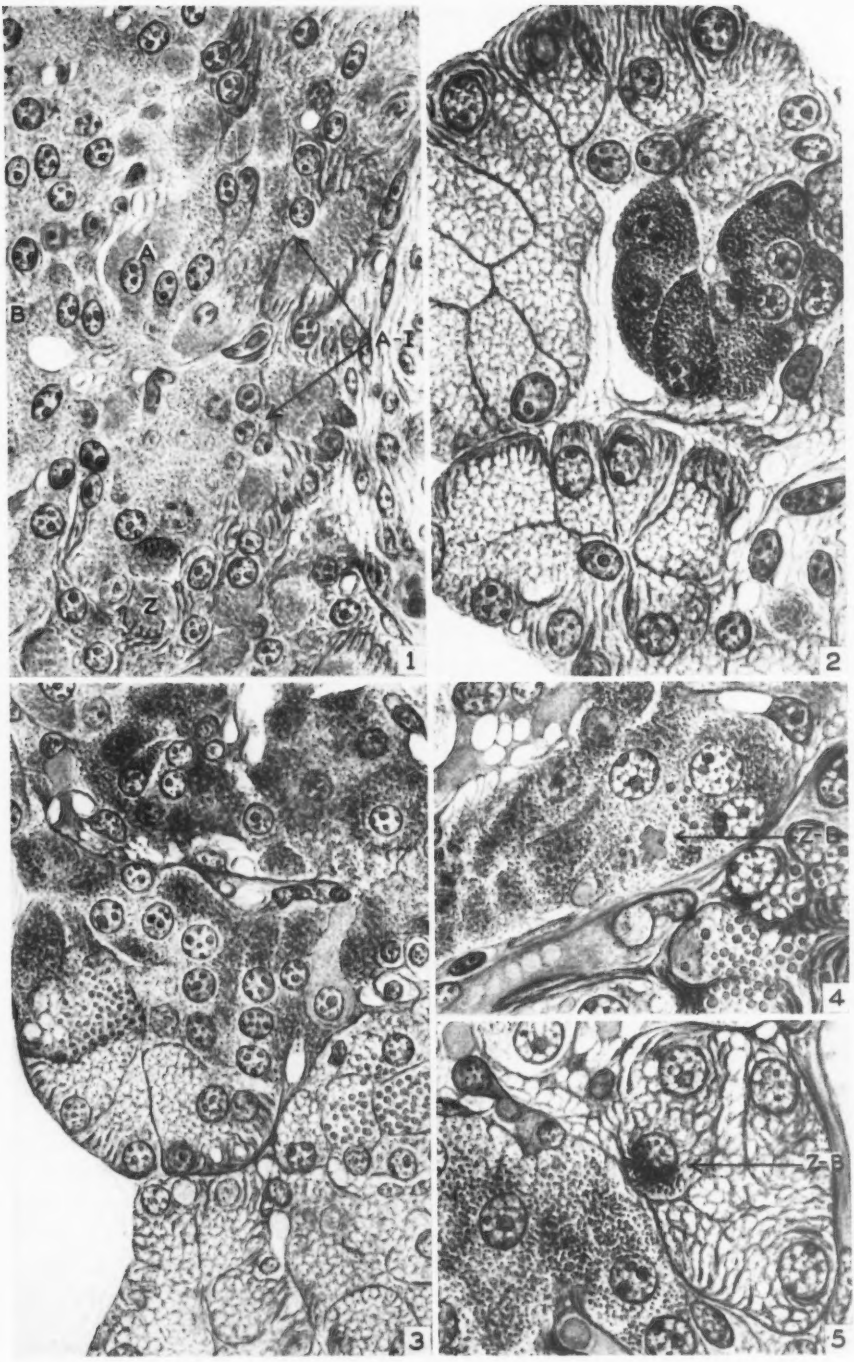
FIG. 1. Section of pancreas from guinea pig sacrificed 4 days after an intravenous injection of 200 mg. alloxan per kilo. Note the vague, irregular boundary between islet (left) and acinar tissue (right); several points of acino-insular transformation (*A-I*) are shown. *A*, A cell; *B*, B cell; *Z*, acinar cell. ×1170.

FIG. 2. Section of pancreas taken from guinea pig 28 hours after alloxan administration (200 mg. per kilo intravenously). The B cells of the newly-formed small islet have retained their acinar arrangement; lumen and some of the cell membranes are still apparent. ×1630.

FIG. 3. Transition cells in the pancreas of a guinea pig sacrificed 24 hours after an injection of alloxan (200 mg. per kilo intravenously). An acinus (left center) at the border of an islet (upper right) shows cells in various stages of redifferentiation into B cells. A centro-acinar cell is seen in the center, and a capillary at the upper right border of this acinus. A few of the acinar cells contain large red zymogen granules. ×1170.

FIG. 4. Section of pancreas from a guinea pig sacrificed 18 hours after injection of alloxan (150 mg. per kilo intravenously). The cell *Z-B* contains both zymogen granules (somewhat coalesced) and B granules; the nucleus of this cell is out of the plane of this section; in an adjacent section it appeared basally to the zymogen granules. The cell to the right of this one contains both intact zymogen granules and B granules. ×1630.

FIG. 5. An acinus (right) adjacent to an islet (left) in the pancreas of a guinea pig given 150 mg. alloxan per kilo intravenously and sacrificed 18 hours later. The cell *Z-B* contains both chromatophilic substance and a clump of B granules partially covering the nucleus. ×1630.




*(See opposite page for legend.)*

sections the connection of the small islets to the ducts could be traced. This is a simple repetition of the embryonic development of the islet tissue (Bensley, 1911; Weichselbaum, 1911), and is not uncommon when the endocrine portion of the pancreas is subjected to additional strain (Florentin and Watrin, 1931; Woerner, 1938; Bakay, 1943; Sendrail, Bazex and Bimes, 1944; Di Pietro, 1945).

The other process by which the B cells are replaced is the transformation of acinar and centroacinar cells into B cells. This mode of origin of islet tissue has been a subject of debate for many years. There is considerable evidence that acinar cells have the potentiality of becoming islet cells and that they may serve as a source of new islet tissue in times of stress (Florentin and Watrin, 1931; Woerner, 1938; De Robertis, 1945; Hughes, 1947; Sergeyeva, 1947). Many of the small islets in the experimental animals which recovered from alloxan administration are in the form and position of acini or parts of acini adjacent to capillaries. The B cells of these islets often have distinct cell boundaries (Figure 2), which is not the case in the untreated guinea pig.

Replacement of B cells in the large islets appears to be chiefly due, also, to transformation of acinar cells. This transformation is evident at the periphery of the islets and gives them a vague outline. Large cells with a few small B granules intermingled with light-staining acinar cells devoid of any zymogen granules and with little basophilic material are a characteristic of the islets during the first 12 to 72 hours after alloxan injection. Hughes (1947) has reported a similar appearance in the rat pancreas following repeated injections of alloxan. Transformation of acinar cells into B cells in the guinea pig following the injection of large amounts of glucose has been described by Woerner (1938).

## SUMMARY

Restoration of the pancreatic islet tissue, which takes place within four days following alloxan administration in the guinea pig, is due to at least three factors: (1) transformation of peri-insular acinar cells by a process of redifferentiation into islet cells; (2) transformation of acini or parts of acini into new small islets; (3) proliferation of the epithelium of the smallest ducts to form new small islets. As judged by the colchicine technique, proliferation of pre-existing B cells plays a very minor role in the replacement of the islet tissue.

Transition cells showing the characteristics of both acinar and B cells are described. The boundaries of many of the islets during and after the recovery phase are indistinct and irregular due to the absence of any connective tissue between the islet and acinar tissue in the regions of peri-insular transformation.

*Acknowledgments*

I wish to express my sincere appreciation to Dr. Philip E. Smith for his suggestions and assistance during the course of this study. Also, I am greatly indebted to Mr. Carl Kellner for preparing the illustrations.

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# FURTHER OBSERVATIONS ON THE INHIBITION OF THE PRODUCTION OF LUTEINIZING HORMONE BY LITHOSPERM

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THE original experiments of Cranston (1945) which demonstrated the presence of an anti-estrus factor in the plant, *Lithospermum ruderale*, served to focus considerable attention on this material. It has been shown that the mechanism of its activity does not involve an inanition effect (Cranston, 1945), a efficiency of the vitamins thiamin, niacin, riboflavin, pyridoxin, or pantothenic acid (Drasher, 1949), or an inhibition of the thyroid (Cranston, 1945; Zahl, 1948; Drasher, 1949). These facts, coupled with the original observation made by Cranston (1945) that the follicle-stimulating hormone would induce estrus in Lithosperm-treated animals, point clearly to a direct inhibitory influence on the production of gonadotropins. Such an overall decrease in gonadotropin potency of the pituitaries of intact mice on a Lithosperm diet was shown by Cranston and Robinson (1949).

The picture becomes somewhat more complex in the castrated animals where, 30 days after castration, no significant difference was observed in either the pituitary weights or the gonadotropin potency between animals receiving normal diet and Lithopserm (Drasher, 1949). It will be recalled that the ovary of the Lithosperm-fed animal does not show a reduction of antra formation. This is good evidence that the FSH out-put of the Lithosperm-treated pituitary is not decreased. These two facts led to the postulation of a differential inhibiting effect upon the luteinizing hormone alone. This is reasonable only if the necessity of the luteinizing hormone for the production of estrogens is accepted.

It seemed necessary to investigate further the behavior of the castrated animal under Lithosperm treatment in order to determine changes, if any, in gonadotropin potency of the pituitaries of animals castrated for perods

Received for publication June 28, 1950.

[1] Contribution #429 from the Department of Zoology, Indiana University. This work was supported by a grant from the Graduate School, Indiana University. Present address: Institute for Cell Research, Karolinska Institute, Stockholm, Sweden.

[2] This is the second paper submitted in partial fulfillment of the requirement for the degree Doctor of Philosophy, Department of Zoology, Indiana University.

[3] The author wishes to express gratitude to Dr. W. R. Breneman of this department for his helpful criticism throughout this work.

longer than 30 days. The rationale behind such investigation was as follows: it has been shown by Hellbaum and Greep (1940) that the luteinizing hormone does not begin to accumulate in appreciable amounts in the pituitary until 20–30 days after castration. It would then be assumed that if Lithosperm were inhibiting LH production alone, such a decrease in gonadotropin potency could be expected only after such a time interval had elapsed. The following experiments on duplicate series of 20, 30, 40, 50 and 60 day castrated females were designed to test this hypothesis.

One of the major difficulties which has beset this work has been the inherent inadequacy of the immature female mouse as an assay animal. The pituitary of the 19 day old female mouse undoubtedly is secreting gonadotropins despite the immature state of its gonads and accessories at this age. Although it is possible to make estimates of the amounts of FSH and LH activity in the material being assayed by combining careful histological examination with organ weight information, this activity of the assay animal's own pituitary invariably introduces undesirable complications. Clark (1935) has reported on investigations of the pituitary potency of young rats where she found a delayed secretory activity of the young male pituitary when compared with females. It seemed that the young male mouse as an assay animal might yield more valuable information than have the females. The use of young males might facilitate the detection of an early inhibition of LH production not recognizable when pituitary material was assayed on immature females. Accordingly, a series of 20 day castrated animals was run on normal and Lithosperm diets for dual assay of their pituitary material on immature males as well as females.

*Effect of Lithosperm on the pituitary potency of normal animals and animals castrated for 20, 30, 40, 50, and 60 days as shown by assay on immature females:*

Young female mice of the Swiss stock of approximately 2½ months of age were employed in this study. In all cases a preliminary control period of at least two weeks was allowed to elapse before experimental procedures were begun, during which period the estrous cycles were checked for normalcy. Vaginal smears were made daily by the lavage method. Castrations were performed bilaterally in one step under Nembutal anaesthesia. Operated animals were allowed a four day recovery period before being placed on the Lithosperm diet.

The Lithosperm[4] was fed at a 20% level mixed with Purina Chow, the whole being fed as pellets prepared by a previously described method

[4] Thanks are hereby extended to Dr. R. A. Diettert of the Botany Department, Montana State University for the collection of this plant material.

(Drasher and Zahl, 1946). Animals received water and diet ad libitum. Animals were sacrificed at appropriate intervals by luxating the cervical vertebrae. The pituitaries were removed immediately for wet weighing followed by dry weighing after desiccation in acetone preparatory to assay. Ovaries, when present, and the uterus plus vagina were wet weighed, these organs being fixed and sectioned for subsequent histological study.

Pituitary assays were done on 19 day old females of the same stock as that of the donors. Animals receiving pituitary powder from intact animals were given the equivalent of two pituitaries, those receiving pituitary powder from castrated animals were given the equivalent of one pituitary. This total dose was administered in six parts at 12 hour intervals for three days. Twenty-four hours were allowed to elapse between the final injection and sacrifice. Ovarian and uterine-vaginal weights were taken and the organs saved for histological study.

The height of the uterine epithelium of the assay animals was measured with an ocular micrometer using oil immersion. The uteri of five animals from each assay group were chosen at random for such measurements, twenty cells being taken from each uterus.

Figure 1 shows the effect of Lithosperm administration on animals for 30 and 60 day periods. This level of Lithosperm can be seen to have effectively inhibited the cycle of the 30 day intact animals (Group B). This effect is not so pronounced in the 60 day intact animals (Group D) although examination of the figure shows that about the same percentage of each group (30%) failed to give an initial response. This percentage corresponds with that determined in previous work (Drasher and Zahl, 1946) for animals of this stock resistant to Lithosperm. Furthermore, in the animals receiving Lithosperm for the 60 day period a refractoriness to the drug was developed as is evidenced by the smear data as well as the organ weights of these animals at autopsy. For this reason pituitary assays were omitted on this group as it was felt that little information pertinant to the particular problem under investigation could be obtained. The refractoriness which develops in animals fed Lithosperm for extended periods remains one of the major difficulties in this work. Pituitary weights of these animals were taken, however, the glands being separated into refractory and non-refractory groups.

Body weights of these animals are shown in Figures 2 and 3. As in previous work the Lithosperm-fed animals show some, but insignificant, weight loss.

Of greater interest are the weights of the pituitaries of animals on normal and Lithosperm diets (Figure 4). Lithosperm administration is seen to decrease markedly both wet and dry pituitary weights in the intact animals. It must be emphasized that the weights shown here for the 60 day

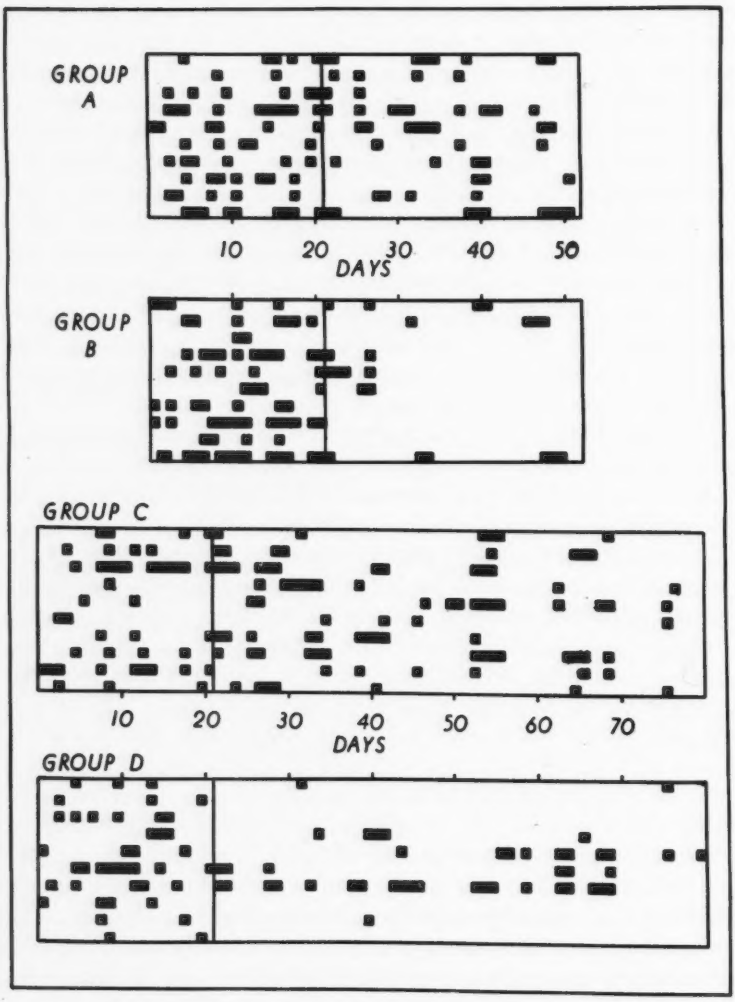


FIG. 1. Vaginal smear data from intact animals on normal and Lithosperm diets. Group A—normal chow, 30 days; Group B—Lithosperm diet, 30 days; Group C—normal chow, 60 days; Group D—Lithosperm diet, 60 days. Blackened squares indicate vaginal cornification. The period before the heavy vertical line indicates preliminary control period.

intact animals receiving Lithosperm are based on only three pituitaries from animals which had not become refractory to the drug, refractoriness being judged on the basis of the vaginal smear data on these animals.

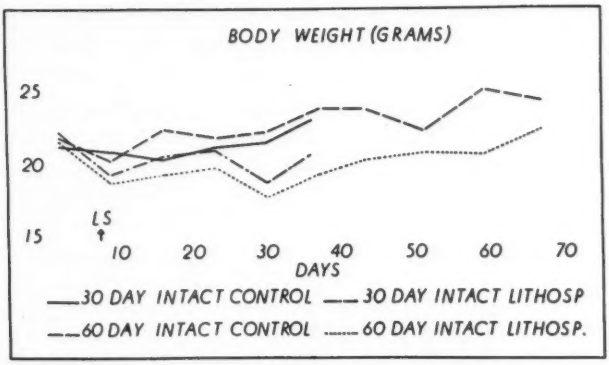


FIG. 2. Body weight data from intact animals on normal and Lithosperm diets for 30 and 60 days.

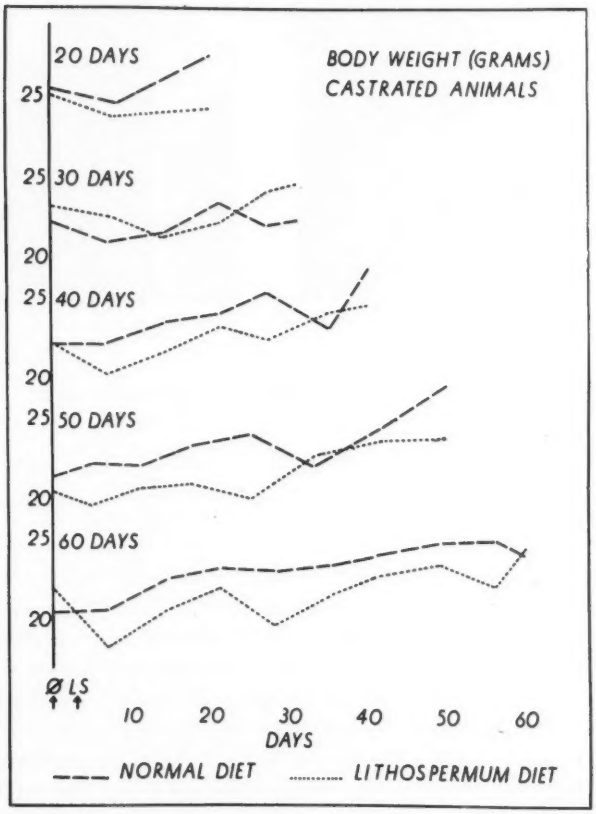


FIG. 3. Body weight data from castrated animals on normal and Lithosperm diets.

Consequently, although the writer feels that this probably represents the true picture for the non-refractory animal, this weight is open to criticism. As will be discussed below, what happens in the animal maintained on Lithosperm for 60 days is not essential for the support of the thesis that Lithosperm acts exclusively as an LH-inhibitor since other proofs are available. In the group of castrated, Lithosperm-fed animals no significant

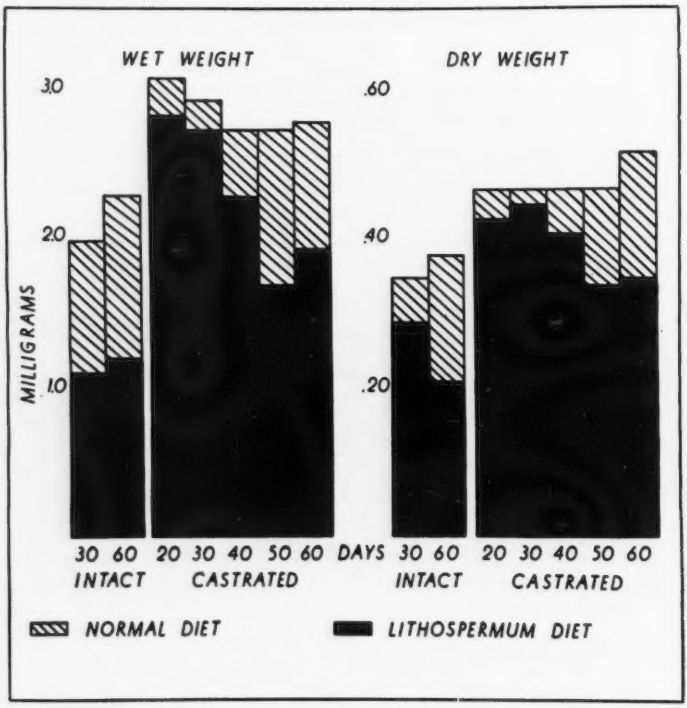


FIG. 4. Dry and wet weights of pituitaries from animals, intact and castrated, on normal and Lithosperm diets.

decrease in pituitary weight is observable until after 30 days. This effect is at its maximum at 50 days and is reflected in the uterine weights of the assay animals receiving these pituitaries (Table 1). The return to apparent normalcy of the pituitary of the 60 day castrated, Lithosperm-fed animal is probably best accounted for, on the basis of present knowledge, by the appearance of refractoriness at this stage. Obviously no effective separation of refractory and non-refractory animals could be made in these castrated groups as had been done in the intact animals using the vaginal smear record. The refractoriness which was obvious in intact animals on Litho-

sperm diet for 60 days makes it necessary to exclude this group of 60 day castrated animals from consideration although the pituitaries of this group were assayed.

The weights of the ovaries were insufficiently sensitive in the assay animals to show significant changes in any case and for this reason have been omitted here. Histologically, however, they show interesting changes indicative of changes in the types of gonadotropins which were introduced. Here, as noted earlier (Drasher, 1949), the donor ovaries of Lithosperm-fed animals gave evidence of depressed luteinization. The ovaries of ani-

TABLE 1. UTERINE WEIGHTS FROM ASSAY ANIMALS RECEIVING PITUITARIES FROM INTACT AND CASTRATED ANIMALS ON NORMAL AND LITHOSPERM DIETS

| Pituitary source | Normal chow | | Lithosperm | |
|---|---|---|---|---|
| | Number of animals | Mean uterine weight | Number of animals | Mean uterine weight |
| Blank | 10 | 15.56 ( .76) | — | — |
| 30 day intact control | 5 | 28.18 (3.08) | 5 | 22.62 (2.35) |
| 20 day castrated | 8 | 26.26 (2.24) | 8 | 31.25* (2.07) |
| 30 day castrated | 8 | 64.72 (2.19) | 8 | 59.74* (2.84) |
| Blank | 10 | 18.29 (1.28) | — | — |
| 40 day castrated | 7 | 50.17 (4.41) | 7 | 41.91* (5.00) |
| 50 day castrated | 8 | 52.54 (3.33) | 8 | 27.94** (2.00) |
| 60 day castrated | 7 | 52.20 (3.92) | 8 | 56.31 (1.72) |

Figures in parentheses following weights are the standard errors of the means. * indicates a significant difference at the 5% level; ** indicates a difference at the 1% level by the "t" test.

mals receiving pituitaries of the intact animals on normal and Lithosperm diets for 30 days showed complete absence of luteinization in all cases. Indeed, no corpora lutea were found in assay ovaries of any group. While the ovaries of animals which received pituitaries from intact animals on normal chow showed no or slight antra formation (indicating a low level of FSH stimulation), those from assay animals which had received pituitaries from intact animals on Lithosperm diet showed large, definite antra (indicating a high level of FSH stimulation). This is to be contrasted with the fact that the reverse is true of the uteri of these animals; those from normal chow group showing evidence of estrogen stimulation while the Lithosperm group gave no such evidence of stimulation. Histologically the vaginae paralleled the uteri rather than the ovaries. A summary of all of these histological data is presented in Table 2. In this connection attention should be drawn to the histological data for the assay animals receiving pituitaries from 20 day castrated animals on normal and Lithosperm diets which points to an increased FSH content of the Lithosperm-fed group

TABLE 2. HISTOLOGY OF OVARIES, UTERI, AND VAGINAE OF ASSAY ANIMALS RECEIVING PITUITARIES FROM INTACT AND CASTRATED ANIMALS ON NORMAL AND LITHOSPERM DIETS

| Pituitary source | Ovaries | | Uteri | | Vaginae |
|---|---|---|---|---|---|
| | Antra | Corpora lutea | Estrogen stim. | Progest. stim. | |
| Blanks | — | — | — | — | — |
| 30 day intact N.C. | * | — | * | — | * |
| 30 day intact LS | ** | — | — | — | — |
| 20 day castrated N.C. | * | — | * | — | * |
| 20 day castrated LS | *** | — | ** | — | ** |
| 30 day castrated N.C. | *** | — | *** | — | ***CC |
| 30 day castrated LS | *** | — | *** | — | ***CC |
| 40 day castrated N.C. | *** | — | ** | — | ***C |
| 40 day castrated LS | *** | — | *** | — | ***CC |
| 50 day castrated N.C. | *** | — | *** | — | ***CC |
| 50 day castrated LS | * | — | * | — | * |

* Indicates degree of character present; C cornification in some animals; CC complete cornification in all animals.

over that of the controls. This effect is also indicated in Table 1 where the uterine weights of this series are slightly heavier in the Lithosperm group.

Results of the histometric analysis of the heights of the uterine epithelia are presented in Table 3 and add confirmation to the above data.

*Effect of Lithosperm on the pituitary potency of 20 day castrated females as shown by assay on immature males:*

A group of 3 month old female Swiss mice was castrated bilaterally and divided into two groups, the pituitaries of Group A to be assayed on immature female mice, and those of Group B to be assayed on immature male mice. These groups were further sub-divided into two sub-groups each, one to receive normal Purina Chow and the other to receive a 20% Lithosperm diet prepared as described previously. At 20 days after castration the animals were sacrificed by a massive dose of Nembutal, decapitated, and the pituitaries removed immediately and wet weighed. They were then desiccated in acetone preparatory to assay.

TABLE 3. HEIGHTS OF UTERINE EPITHELIUM FROM ASSAY ANIMALS RECEIVING PITUITARIES FROM INTACT AND CASTRATED ANIMALS ON NORMAL AND LITHOSPERM DIETS (MICROMETER UNITS)

| Pituitary source | Normal chow Mean height | Lithosperm Mean height | d.f. | t-value |
|---|---|---|---|---|
| Blank | 12.08 (.19) | — | | |
| 30 day intact | 14.52 (.23) | 12.26 (.21) | 198 | 2.29* |
| 20 day castrated | 14.12 (.29) | 16.10 (.29) | 198 | 1.56 |
| 30 day castrated | 22.77 (.53) | 19.34 (.47) | 198 | 1.56 |
| 40 day castrated | 19.39 (.46) | 21.34 (.52) | 198 | .90 |
| 50 day castrated | 22.36 (.41) | 14.20 (.24) | 198 | 5.48** |

Figures in parentheses following heights are the standard errors of the means. * indicates a significant differences at the 5% level; ** a difference at the 1% level by the "t" test.

Nineteen day old animals received the equivalent of two pituitaries given subcutaneously in six injection of .25 cc. each over a period of three days, a total of 1.5 cc. per animal. The animals were sacrificed 24 hours after the last injection by dislocation of the cervical vertebrae. The organs were removed and wet weighed and then fixed in Bouin's fluid for subsequent histological study. The organ weights of these animals are shown in Table 4.

An inspection of Table 4, Group A (Female assay animals) serves to confirm observations made earlier (Drasher, 1949) that Lithosperm administration to animals castrated 20 days results in no apparent decrease

TABLE 4. ORGAN WEIGHTS OF MALE AND FEMALE ASSAY ANIMALS RECEIVING PITUITARIES FROM 20 DAY CASTRATED FEMALE MICE ON NORMAL AND LITHOSPERM DIETS

Group A—female assay animals

| Pituitary source | Number of animals | Ovaries (mg.) | Uterus vagina (mg.) |
|---|---|---|---|
| Blank | 9 | 5.58 ( .53) | 16.93 (1.03) |
| Normal Chow | 10 | 8.14** ( .43) | 57.44** (2.51) |
| 20% Lithosperm | 10 | 9.30** (1.02) | 62.34** (1.56) |

Group B—male assay animals

| Pituitary source | Number of animals | Testes (mg.) | Seminal vesicles (mg.) | Ventral prostate (mg.) |
|---|---|---|---|---|
| Blank | 9 | 48.02 (2.42) | 4.09 (.26) | .93 |
| Normal Chow | 9 | 55.51 (3.77) | 10.67**## (.75) | 2.3 |
| 20% Lithosperm | 9 | 52.00 (3.39) | 5.47** (.41) | 1.1 |

Figures in parentheses following weights are standard errors of the means. The weights of the ventral prostates are from pooled weighings and have not been treated statistically. ** indicates significance at the 1% level in comparison with the Blank by the "t" test; ## a difference at the 1% level in comparison with the 20% Lithosperm group.

in pituitary potency. Here again there is indication of increased pituitary activity in the Lithosperm-treated group although these differences are not statistically significant for either ovarian or uterine weights. The same type of pituitary material, when assayed on immature male mice (Table 4, Group B), however, yields surprisingly different results. These animals, while showing no significant changes in testis weight, effectively show the depressing effect of Lithosperm on the pituitary LH content by the depressed effect on androgen production by the testes of the male assay animals where there are significant decreases in both seminal vesicle and ventral prostate weight in comparison with animals treated with normal pituitary material. Obviously, there is a considerable difference in the response given by the male and female assay animals to the same pituitary preparations. The results of histological examination of the

gonads and accessories of these assay animals are shown in Table 5. The histology of the testes of the male assay animals was not helpful. Weight data on the accessory structures, such as the seminal vesicles and ventral prostates, clearly indicate that androgens were not being produced to any great degree by the testes of assay animals receiving pituitary material

TABLE 5. COMPARISON OF THE HISTOLOGY OF THE GONADS OF THE FEMALE ASSAY ANIMALS RECEIVING PITUITARIES FROM 20 DAY CASTRATED FEMALE MICE ON NORMAL AND LITHOSPERM DIETS

| | Blanks | | Normal chow | | 20% lithosperm | |
|---|---|---|---|---|---|---|
| | Antra | Corpora lutea | Antra | Corpora lutea | Antra | Corpora lutea |
| #1 | — | — | * | **** | ** | — |
| #2 | — | — | ** | — | ** | — |
| #3 | — | — | ** | ** | ** | — |
| #4 | * | — | * | — | ** | — |
| #5 | — | — | ** | **** | ** | — |
| #6 | * | — | ** | *** | * | — |
| #7 | — | — | ** | — | * | — |
| #8 | — | — | ** | — | ** | — |
| #9 | — | — | ** | **** | *** | — |
| #10 | | | * | — | ** | — |

* Indicates degree of character present; — absence of character.

from castrated donors on Lithosperm diet in contrast with the amounts of androgens produced by the assay testes stimulated by pituitary powder from castrated donors on normal diet. However, no differences in the amounts of interstitial cells of the testes could be detected. Results of the measurements of the cell heights of the seminal vesicle and uterine epithelium are indicated in Table 6. Heights of the cells of the epithelium of

TABLE 6. HEIGHTS OF THE EPITHELIUM OF SEMINAL VESICLES AND UTERI OF ASSAY ANIMALS RECEIVING PITUITARIES FROM 20 DAY CASTRATED FEMALE MICE ON NORMAL AND LITHOSPERM DIETS (MEASUREMENTS IN MICROMETER UNITS UNDER OIL IMMERSION)

| Pituitary source | Seminal vesicles | Uteri |
|---|---|---|
| Blank | 8.69 (.152) | 13.50 (.237) |
| 20 day castrated Normal Chow | 9.81** (.183) | 22.82** (.394) |
| 20 day castrated 20% Lithosperm | 8.60 (.149) | 21.51** (.347) |

Values given represent means of 100 measurements, 20 cells being measured at random over the glands from 5 animals in each group. Figures in parentheses following the means are standard errors of the mean. ** indicates statistical significance beyond the 1% level by the "t" test.

the ventral prostates could not be measured in the case of glands from animals which had received pituitaries from animals on normal chow. In this assay series, the stimulation of the epithelium was so intense that the lumen of the ventral prostate lobules was obliterated almost completely.

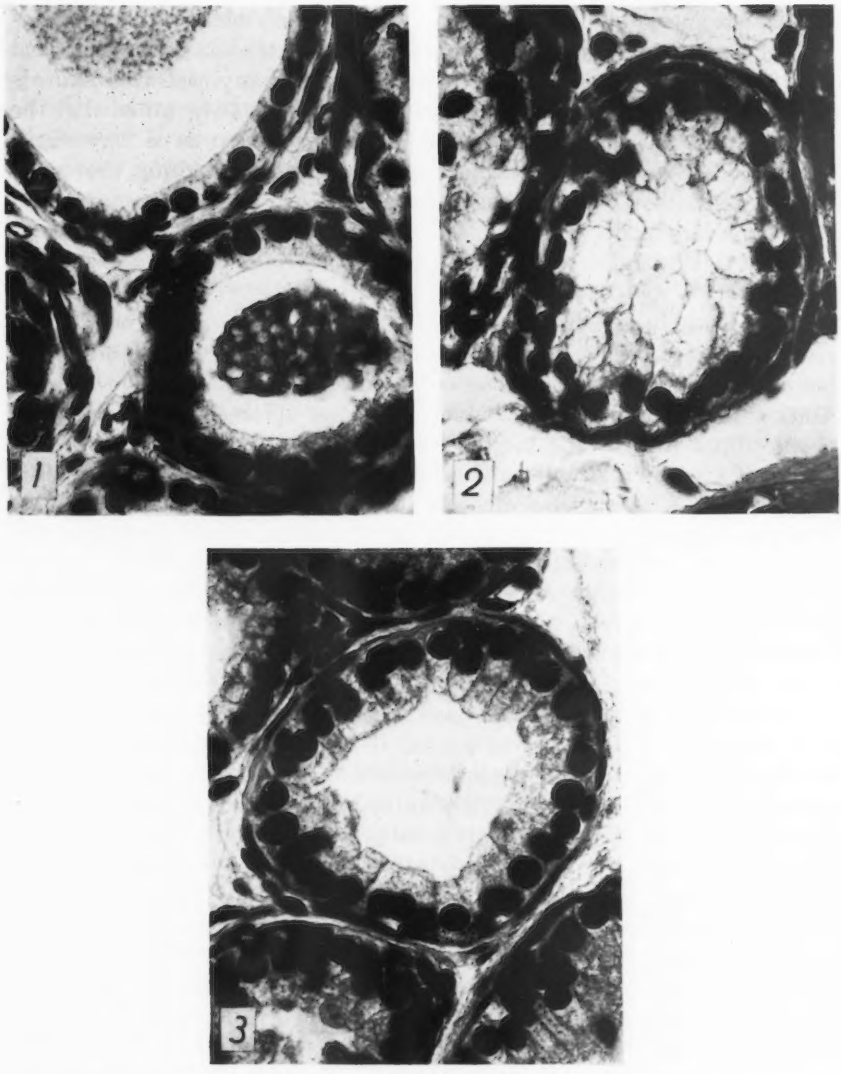


FIG. 5. Photomicrographs of representative sections of the ventral prostates of male assay animals. #1. Gland from blank assay animal receiving injections of distilled water. #2. Gland from assay animal receiving two pituitary equivalents from 20 day castrated animal on normal chow. #3. Gland from assay animal receiving two pituitary equivalents from 20 day castrated animal on 20% Lithosperm diet. Bouin fixation, cut at 5 micra, stained with Delafield's hematoxylin and eosin-azure. Magnification ×900.

Photomicrographs of representative sections from blank assay animals (#1), those receiving pituitaries from 20 day castrated animals on normal chow (#2), and those receiving pituitaries from 20 day castrated animals on Lithosperm diet (#3) are shown in Figure 5. It is to be noted that the epithelium of the Lithosperm group (#3) shows evidences of very slight stimulation whereas the epithelium of the normal diet group shows extreme stimulation. These findings substantiate the weight data presented in Table 4, Group B.

## DISCUSSION

On the basis of earlier work of Hellbaum and Greep (1940) on the rat, it was suggested previously that the slight effect of Lithosperm on the 30 day castrate pituitary of the mouse in contrast to its marked effect in the intact animal might be due to a corresponding differential change in the gonadotropin titer of the mouse pituitary after castration. This would consist of a preliminary elevation of FSH followed by a rise in LH until after 30 days LH would become the dominant gonadotropin of the castrate pituitary. Consequently, it was expected that if such were the case in the normal castrated mouse and that if Lithosperm were inhibiting LH production alone, Lithosperm should have a pronounced effect in reducing pituitary potency in the castrated mouse sometime after 30 days.

The above results indicate that such is the case. Indeed, contrary to results obtained earlier (Drasher, 1949) some decrease in gonadotropin potency is already evident at 30 days although this is statistically significant at only the 5% level using the "t" test. Obviously the exact time at which this decrease becomes apparent would be expected to vary somewhat depending on the individual group of animals used and on the sensitivity of the assay animals. This decrease is more pronounced at 40 days and by 50 days after castration is highly significant falling far beyond the 1% level of probability.

An examination of Table 1 and 2 reveals an interesting anomaly for the pituitary potency of 20 day castrated animals. Here the effect of Lithosperm seems to be reversed, tending to increase gonadotropin titer. Whether this is a real effect must await futher investigation. If true, it indicates an augmentation effect of Lithosperm on the production of FSH, at least at the early stages of administration. By 30 days after castration, when LH is becoming the predominant gonadotropin of the castrate pituitary this augmentation of FSH would be expected to become obscured and would no longer be obvious in the assay material. That this may actually be the case is indicated also by the histological picture presented by the organs of the assay animals receiving pituitaries from intact animals on nor-

mal and Lithosperm diets for a period of 30 days. While the uteri and vaginae of animals receiving pituitary powder from Lithosperm-treated animals showed complete absence of estrogenic stimulation, the ovaries of these same animals contained large, well-formed antra, indicative of marked FSH stimulation. On the other hand, animals which were given pituitary powder from donors on normal chow showed slight FSH stimulation of the ovaries but did possess accessory organs which definitely were being stimulated by estrogens.

These facts can be interpreted only in the light of the conclusions drawn by Greep, van Dyke and Chow (1942) which state that FSH stimulation alone can not result in estrogen production although it does stimulate antra production in the ovary. This, then, may account for the increased antra formation in the ovaries of assay animals receiving pituitaries from intact, Lithosperm-fed animals which was unaccompanied by estrogen stimulation of the accessories. Normal diet animals of the same series, whose pituitaries did not have a Lithosperm-induced augmentation of FSH, did however, possess their normal complement of LH and were able to induce estrogen secretion by the assay ovaries before histological effects of such stimulation occurred. It is well to emphasize at this point that ovarian histology alone is a poor criterion for determining the type of hormonal secretion being produced by that gland and must be considered in connection with effects produced in the secondary sex structures since premorphological secretion of estrogen is common. Similarly the weights of these organs is not always definitive in determining the exact nature of the stimulation being received.

Let us turn now to the results obtained on the pituitary potency of animals castrated for 20 days and assayed on immature males as well as females. It will be recalled that this same type of pituitary material (from 20 day castrated females on normal and Lithosperm diets) yielded astonishingly different results (Table 4). Using the male assay animal a definite depression of LH content of the pituitary when treated with Lithosperm could be detected. These differences between the responsiveness of male and female assay animals have been interpreted as indicating an earlier secretion of gonadotropins by the female pituitary so that in immature females the pituitaries are already producing sufficient LH to result in estrogen secretion by the ovaries when sufficient FSH from an exogenous source (donor pituitaries in this case) is present. The male pituitary, on the other hand, at 21 days of age is not yet producing ample LH to result in the production of androgens by the testes unless LH as well as FSH from an exogenous source is added. These conclusions corroborate the observations of Clark (1935) on the rat for the ages at which pituitary

secretion begins in males and females. Results of pituitary assays using the immature male mouse, then, confirm the idea proposed earlier that Lithosperm inhibits the production of LH only.

Histological examination of the gonads and accessories (Table 5) of these assay animals yielded more conclusive results on the differential inhibition of LH by Lithosperm. Previous assays of similarly treated pituitary material in which one pituitary equivalent was administered from castrated animals (Table 2) resulted in no luteinization in the ovaries of any group. At a level of two pituitary equivalents luteinization was produced in the majority of the female assay animals receiving pituitary material from 20 day castrated female animals on normal diet. Such was not the case in the assay animals receiving pituitaries from similar animals maintained on a 20% Lithosperm diet despite the fact that these ovaries were somewhat heavier than those from the normal diet group. The accessories of both groups of female assay animals showed evidence of estrogenic stimulation (in itself evidence of LH stimulation of the gonads). It will be recalled, however, that the pituitaries of the female assay animals are, by the age of assay, secreting small amounts of LH, which can account for this secretion of estrogenic substances in amounts sufficient to bring about the development of the uterus and vagina. This endogenous LH is not sufficient to cause luteinization of the ovaries unless large amounts of LH are introduced from an exogenous source (the pituitary material being assayed in this case). At a level of administration of one pituitary equivalent, the LH content of the pituitary of the castrated animal on normal chow is not sufficient to bring about a luteinization response. Using a two pituitary equivalent dose, as has been done in this second series, gross luteinization of the recipient ovaries could be produced. This luteinization of the assay ovaries occurred only in those ovaries subjected to stimulation by pituitary material from castrated animals on normal chow and not by material from animals maintained on a 20% Lithosperm diet. Thus, despite the small amounts of endogenous LH being produced by the pituitaries of the female assay animals themselves, it has been possible to differentiate between the almost pure FSH stimulation produced by pituitaries from Lithosperm-treated animals and the mixed FSH and LH stimulation by pituitaries from animals on normal diet.

It is suggested that for work involving the estimation of relative amounts of FSH and LH in pituitary preparations of this type the combined use of both female and male assay animals is superior to the use of either alone. Histological changes in the ovaries at pituitary dosage insufficient to produce luteinization offers a means of comparing FSH levels in the preparation, whereas higher dosages will allow the detection of LH in the preparation. The delayed secretion of LH in the young male makes it a better

assay animal for LH levels if the preparation contains FSH as well, by a comparison of the weights of the accessories. The immature male mouse is however, very poor as a means of detecting FSH alone because of the lack of easily recognizable histological changes in the testes.

## SUMMARY

*Lithospermum ruderale* was fed to intact and castrated female mice. Castrated animals were sacrificed at 10 day intervals beginning at 20 days and extending to 60 days. Lithosperm administration was found to diminish gonadotropin titer in a highly significant manner only after 40 days of castration when the female assay animal was used. This effect reached a maximum at 50 days. By 60 days the animals had apparently become refractory to the drug and exhibited a return to normal post-castration potency. On the basis of the number of animals which became refractory in the intact 60 day group, results of the assay of the pituitaries of this 60 day castrated group on Lithosperm must be considered as inconsequential for the present purpose.

Pituitary material from 20-day castrated female mice on Lithosperm when assayed on males and females at a higher pituitary equivalent dose showed marked depression of LH content alone. This was shown by an absence of luteinization in the ovaries of assay females receiving pituitary material from animals maintained on Lithosperm and the absence of significant stimulation of the accessory structures of the male assay animals receiving this same pituitary material.

The hypothesis of Lithosperm action by a direct and differential depression of the production of the luteinizing hormone alone would then seem to be substantially strengthened by these results.

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# THE EFFECTS OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE UPON THE ADRENALS OF NORMAL AND OF HYPOPHYSECTOMIZED RATS MAINTAINED WITH ADRENOCORTICOTROPIN[1]

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## INTRODUCTION

THE authors working with De Majo (1949) showed that methyl testosterone, vinyl testosterone and testosterone propionate in a dosage of 2.5 mg. daily for 20 days and desoxycorticosterone acetate or progesterone at the level of 5 mg. daily for the same period of time produced a significant decrease in the size of the adrenals of normal female rats. No such atrophy was obtained with comparable doses of dehydroisoandrosterone, ethynyl testosterone or ethyl testosterone. It was noted in the course of these studies that testosterone propionate and methyl testosterone caused a decrease, whereas progesterone, desoxycorticosterone acetate and vinyl testosterone produced an increase in the cholesterol concentration of the adrenals.

Wells and Kendall (1940) showed that the administration of 1 mg. daily of 11-dehydro-17-hydroxycorticosterone (Cpd. E) to normal rats produced a suppression of growth and a marked decrease in the size of the adrenal glands. More recently Stebbins (1950) showed that a dose of 3 mg. of Cpd. E given to normal rats for 42 days produces atrophy of all the cortical zones except the glomerulosa.

Sayers and Sayers (1948) have shown that Cpd. E and related steroids inhibit the release of adrenocorticotropic hormone (ACTH) which usually follows stress stimuli. They suggest that the anterior pituitary is influenced in its secretory activity by a deficiency or excess of cortin. However their findings do not rule out the possibility that Cpd. E might exert a direct effect upon the adrenal cortex. Such a direct action of testosterone upon

Received for publication June 29, 1950.

[1] This work was made possible by a grant from the American Cancer Society for studies on the relationship of the pituitary hormones, thyroid hormone and the steroid hormones of the adrenal glands and gonads to normal and abnormal growth.

[2] Argentine Fellow of the Mead Johnson Fellowship of the Society for Pediatric Research.

the adrenals of hypophysectomized rats has been observed by Lewis, De Majo and Rosemberg (1949) and upon the x-zone of the adrenals of hypophysectomized mice by Jones (1950).

The following experiment was devised to elucidate the relative importance of the pathways through which Cpd. E causes a reduction in adrenal size and functional activity as measured by the cholesterol concentration.

## METHODS

Twenty hypophysectomized[3] female albino rats 8 weeks of age were fed Gaine's Dog Kruncheon supplemented by milk and bread while a similar group of 20 normal female albino rats 8 weeks of age were maintained on Dog Kruncheon and tap water.

All of the hypophysectomized animals were given 3 injections daily of 0.4 mg. ACTH[4] for the 20 days of the experiment. Half of the hypophysectomized rats and half of the normal animals were injected once daily with 1.25 mg. of an aqueous suspension of Cpd. E acetate[5] for the 20 days.

The rats were autopsied at 11 weeks of age. The organs were weighed on a torsion balance, the right adrenal placed in Bloor's solution and the left adrenal preserved in fixative for histological study. From each of the four groups of animals it was possible to obtain two cholesterol determinat ons by pooling 5 adrenals which were ground and extracted. The cholesterol concentration was determined according to the method described by Peters and Van Slyke (1932).

## RESULTS

The effect of 20 daily injections of 1.25 mg. Cpd. E acetate upon body weight, adrenal weight and adrenal cholesterol concentration are shown in Table 1. The normal controls gained 20 grams of weight while the nor-

TABLE 1. EFFECT OF CPD. E UPON BODY WEIGHT, ADRENAL WEIGHT AND ADRENAL CHOLESTEROL CONCENTRATION OF NORMAL RATS AND HYPOPHYSECTOMIZED RATS MAINTAINED WITH ACTH

| | Number animals in group | Initial body weight, gm.* | Final body weight, gm.* | Adrenal weight, mg.* | Average adrenal cholesterol concentration, mg./100 mg. |
|---|---|---|---|---|---|
| Normal controls | 10 | 160 (2) | 180 (4) | 33.8 (1.2) | 5.2 |
| Normal Cpd. E | 10 | 161 (3) | 155 (4) | 22.9 (1.4) | 7.2 |
| Effect of Cpd. E in normals | | | −25 (6) | −10.9 (1.9) | 2.0 |
| Hypophysect. with ACTH controls | 10 | 137 (2) | 125 (2) | 26.7 (1.4) | 11.8 |
| Hypophysect. with ACTH Cpd. E | 10 | 138 (2) | 101 (2) | 27.9 (1.8) | 11.6 |
| Effect of Cpd. E in hypophysect. with ACTH | | | −24 (3) | 1.2 (2.3) | −0.2 |

* Standard Deviation given in parenthesis.

[3] Hypophysectomized rats and normal animals of the same age were obtained from Hormone Assays, Chicago, Illinois.

[4] The ACTH used in this experiment was kindly supplied by the Armour Laboratories Inc., Chicago, Illinois.

[5] The Cpd. E acetate was supplied by Merck and Co., Rahway, New Jersey.

mal rats treated with Cpd. E lost 6 grams of weight. The adrenal glands of the normal rats treated with Cpd. E were 32 per cent smaller than the normal controls although the reduction was only 21 per cent when the adrenal gland weight was calculated in terms of the final body weight. At the same time that the adrenal size was reduced the cholesterol concentration of the adrenals rose almost 40 per cent. Histological observations will be reported elsewhere.

The hypophysectomized rats which received ACTH but not Cpd. E

TABLE 2. CHANGE IN ADRENAL WEIGHT AND ADRENAL CHOLESTEROL CONCENTRATION FOLLOWING TREATMENT WITH VARIOUS STEROIDS

| Steroid injected | Daily dose, mg. | Change in adrenal weight, %* | Change in adrenal cholesterol concentration, % |
|---|---|---|---|
| methyl testosterone | 2.5 | −39 | −23 |
| | 5.0 | −24 | −54 |
| desoxycortico-sterone acetate | 1.25 | − 5 | 0 |
| | 2.5 | − 5 | 17 |
| | 5.0 | −30 | 19 |
| progesterone | 1.25 | − 8 | − 5 |
| | 2.5 | − 5 | −21 |
| | 5.0 | −25 | 15 |
| vinyl testosterone | 1.25 | − 4 | 11 |
| | 2.5 | −28 | 22 |
| | 5.0 | −36 | 16 |
| pregnenolone | 2.5 | −12 | − 1 |
| testosterone propionate | 2.5 | −37 | −24 |
| 11-dehydro-17-hydroxy-corticosterone, Cpd. E | 1.25 | −21 | 39 |

* Adrenal weight ratio to final body weight.

lost 12 grams of weight during the experiment while these that received both ACTH and Cpd. E lost 37 grams. The adrenal size and cholesterol concentration in the hypophysectomized animals treated with ACTH was the same whether Cpd. E was administered or not.

For purposes of comparison the results obtained in this experiment are listed in Table 2 with those previously obtained (1949). Of those steroids tested in the dose level of 1.25 mg., Cpd. E was the only one which produced a significant reduction in adrenal size. It should also be noted that the increase in adrenal cholesterol concentration produced by 1.25 mg. of Cpd. E was greater that the increase following the administration of any other steroid regardless of the dose employed.

## DISCUSSION

The growth retarding effect of Cpd. E originally noted by Wells and Kendall (1940) was apparent in both the normal rats and those which were hypophysectomized and treated with ACTH. It is obvious that this is due to a direct effect of Cpd. E on the body metabolism.

Although there was decrease in the weight of the adrenals and increase in their cholesterol concentration in normal rats treated with Cpd. E, this was not so when Cpd. E was given to hypophysectomized animals maintained on a constant dose of ACTH. We feel that this is definite evidence in favor of the hypothesis that the adrenal atrophy produced by treatment with Cpd. E is due entirely to suppression of pituitary secretion of ACTH.

Our observations suggest that the beneficial effects which have been observed in the treatment of adrenal hyperplasia with Cpd. E, Wilkins, Lewis, Klein and Rosemberg (1950) are the result of diminished production of ACTH.

## SUMMARY

The daily administration of 1.25 mg. of Cpd. E acetate for 20 days to normal female rats causes a significant reduction in adrenal size and rise in adrenal cholesterol concentration. Similar treatment of hypophysectomized female rats maintained with ACTH does not effect the adrenal size or cholesterol concentration. Adrenal atrophy produced by treatment with Cpd. E is caused chiefly if not entirely by inhibition of the production of ACTH.

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# OXIDATION OF 11-DESOXYCORTICOSTEROIDS WITH ADRENAL TISSUE HOMOGENATES[1,2]

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IN THE course of the past year it has been demonstrated that the isolated perfused adrenal gland is capable of specific oxygenation of the steroid molecule in the carbon-11 position. Perfusion of the intact beef adrenal with serum or whole blood in the presence of adrenocorticotrophic hormone results in the appearance in the perfusate of pronounced adrenal cortical hormone activity (Hechter, 1949). Studies in which 11-desoxycorticosterone, 17-hydroxy-11-desoxycorticosterone and androsterone were added to the perfusing fluid resulted in the specific oxidation of the steroids at the carbon-11 position, forming corticosterone, 17-hydroxycorticosterone and 11-($\beta$)-hydroxyandrosterone respectively (Hechter, Jacobsen, Jeanloz, Levy, Marshall, Pincus and Schenker, 1949 and 1950).

As a result of these studies by the Worcester group, the idea has arisen in a number of laboratories including our own, that oxidation of the steroid molecule at carbon-11 might be accomplished by the use of homogenates and perhaps cell-free extracts of adrenal tissue. Hayano, Dorfman and Prins (1949) have in fact reported that incubation of desoxycorticosterone with adrenal tissue homogenates results in the appearance of significantly increased levels of glycogenic activity. We now report a continuation of this study and its extension to the incubation of 17-hydroxy-11-desoxycorticosterone. Examination by means of paper chromatographic technique of the biologically active extracts from the incubation of both these steroids indicates that corticosterone and 17-hydroxycorticosterone are formed from the respective 11-desoxy compounds in concentrations which account for the total glycogenic activity formed during the respective incubations.

## EXPERIMENTAL

*Incubation:* Intact adrenal glands, collected and frozen in dry-ice at the slaughterhouse were homogenized on the same day as collection, in a Waring blender in a phos-

Received for publication July 5, 1950.

[1] Presented before the 34th annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, April 18th, 1950.

[2] This investigation was supported (in part) by a research grant from The National Heart Institute of the National Institutes of Health, Public Health Service.

phate-physiological saline medium (0.83% sodium chloride, 0.084% potassium chloride and 0.015% magnesium chloride in 0.02 M phosphate buffer) at neutral pH. The ratio of 25 g. of adrenal tissue to 100 ml. of buffer solution was maintained in all incubations. To this homogenate the steroid (20 mg. of desoxycorticosterone or 30 mg. 17-hydroxy-11-desoxycorticosterone acetate) was added, dissolved in 1.0 ml. of ethyl alcohol or propylene glycol. Modification of the incubation medium was made by replacing part of the buffer solution with an equal volume of beef blood, collected and defibrinated on the day of use. Addition of substances (ascorbic acid, riboflavin, glutathione) to the medium was made in attempts to enhance production of glycogenic material. Several incubations were carried out using two water soluble forms of desoxycorticosterone, one a special aqueous preparation of the acetate[3] and the other the glucoside. The addition of dihydrostreptomycin hydrochloride (1.0 mg. per 100 ml.) suppressed bacterial growth.

The mixtures were allowed to incubate in an air oven at 37° C. with occasional stirring by hand. At the end of the incubation period ethyl alcohol (95%) was added to a final concentration of 70%.

*Extraction of steroids:* The mixture resulting from the addition of alcohol to the incubation homogenate was allowed to stand overnight at room temperature and filtered. After washing the protein precipitate and tissue debris with seventy per cent ethyl alcohol, the final volume of the combined solutions totalled approximately 700 ml.; these were extracted three times with 100 ml. portions of petroleum ether to remove the major part of the fat and cholesterol. These extracts were combined and extracted with 30 ml. of seventy per cent alcohol. The combined alcoholic solutions were evaporated to small volume to remove all traces of alcohol and the residual solution diluted to 100 ml. with water. All evaporations were carried out under reduced pressure under an atmosphere of nitrogen at temperatures below 37°C. The aqueous solution was extracted with three 100 ml. volumes of ethylene dichloride. The combined ethylene dichloride extracts were washed once with 30 ml. of water, dried over sodium sulfate and evaporated to dryness. The residue was dissolved in 100 ml. of ethyl acetate and the solution washed successively five times with 10 ml. of one per cent sodium carbonate, once with 10 ml. of water, five times with 10 ml. of N/2 hydrochloric acid and finally with one 10 ml. volume of water, dried over sodium sulfate and evaporated to dryness. This procedure was effective in removing all traces of adrenalin as measured by the fluorescence in alkaline medium. The residue from the ethyl acetate solution, weighing between 30 and 45 mg., consisted of approximately fifty per cent steroid material as measured by the ultraviolet absorption at 240 mμ. This material was dissolved in a convenient volume of ethyl alcohol and was found suitable for chemical chromatography or biological assay.

*Biological Assay:* The glycogen deposition method of Venning, Kazmin and Bell (1946) was employed using adrenalectomized Rockland Farm mice weighing 18–25 g. Liver glycogen was determined by the method of Good, Kramer, and Somogyi (1933). An interval of three to four days was allowed between time of arrival and adrenalectomy to permit the animals to adapt to laboratory conditions. Preparations resulting from the incubation studies were administered in 10% ethyl alcohol physiological saline solution and were at all times assayed simultaneously with an aqueous adrenal extract (Upjohn) which was employed as comparative standard. This standard adrenal extract was assayed and found to contain per ml. glycogenic activity equivalent to 400–500 μg. of corticosterone or 125–150 μg. of 17-hydroxy-11-dehydrocorticosterone acetate.

*Chromatographic analysis:* The method employed was described by Zaffaroni, Burton and Keutman (1950). Filter paper strips were impregnated with formamide or propylene

[3] Organon, Inc.

glycol and the alcoholic solution of the extracted steroids applied to the paper strip in a stream of air directed at the point of application. The strips were suspended in an air-tight container and benzene or toluene allowed to flow downward over the paper from a trough-like container. After a suitable length of time the paper strips were removed and dried. The chemical reaction used to determine the position of the separated steroids was that outlined by the same authors, consisting of immersing the strips in a solution of ammoniacal silver hydroxide until complete reaction (usually about two minutes) followed by fixing in sodium thiosulfate solution and washing in water. In larger preparative chromatograms, filter paper sheets 16 cm. wide and 42 cm. long were used instead of the strips. Volumes of 0.3 ml. containing between 4 and 5 mg. of total steroid mixture were applied along a line 4–5 cm. from the top of the sheet. These were placed in a trough and developed in the usual fashion; after removal of the solvents by drying, strips 1 cm. wide were cut from the lateral edges and subjected to the chemical development. After drying, the cut strips were returned to their positions on the original sheets and the areas of the separated steroids located by the position of the darkened zones in the two chemically treated side strips.

## RESULTS

*Biological:* It is important to point out that even under optimum conditions not all experiments resulted in the formation of glycogenic material. The reason for these occasional failures is not understood. The discussion which follows concerns only those preparations showing enhanced biological activity. Furthermore, the problem of assay was complicated by the presence in the incubation extracts of unknown material toxic to adrenalectomized mice. Such extracts at high dosage levels killed all animals; intermediate levels caused the animals to appear ill with little glycogen in the livers and at low dosage levels produced good glycogen deposition. Only when the glycogenic activity was high enough to permit administration of an extract at low concentrations was the toxic material sufficiently diluted to provide satisfactory dose-response relationships. The chemical purification of the extracts described above was only slightly effective in alleviating this problem. The toxic material was however very effectively removed, presumably by adsorption, when the ethyl alcohol extracts were subjected to chromatographic separation on paper. This is illustrated in the glycogenic titers of extracts of 17-hydroxy-11-desoxycorticosterone incubations before and after chromatography, listed in the right hand column of Table 1. A further problem inherent in the assay itself, was the large variation, from day to day, in the biological response to samples of equal corticosteroid content. This is attributable to the variable stresses to which the mice were subjected during transit to our laboratories. It was controlled by comparison with the standard adrenal extract response curve, determined simultaneously with the unknown extracts.

Incubation of 25 g. of homogenized adrenal tissue for four hours without added steroid substrate gave extracts whose glycogen assay values, expressed in terms of ml. of the standard adrenal extract (Upjohn) are shown in column 1 of Table 1; these values serve as the baseline of glycogenic

steroid content of the adrenal tissue employed in the incubations. The inclusion of defibrinated blood in the medium did not alter this value and such controls are included in those shown in Table 1. Nor does there appear to be any important difference between the values obtained when the controls were allowed to incubate and when they were not (Figure 1). The second column of Table I shows the values obtained when 20 mg. of desoxy-

TABLE 1. GLYCOGENIC ACTIVITY RESULTING FROM INCUBATION FOR FOUR HOURS OF 11-DESOXYCORTICOSTEROIDS WITH HOMOGENATES OF ADRENAL TISSUE
(Activity expressed as ml. equivalents of a standard adrenal extract*)

| Control | 20 mg. desoxycorticosterone | | 30 mg. 17-hydroxy-11-desoxycorticosterone acetate |
|---|---|---|---|
| Buffer medium | | | Blood buffer medium |
| — | 8.5 | 17.7 | — |
| 1.7 | 6.8 | — | — |
| 1.4† | 5.3 | 15.4 | — |
| 2.3 | 6.3 | — | — |
| 2.1† | 4.2 | 14.7 | — |
| 2.0†‡ | — | — | 52.5 before chromatography<br>77.0 after chromatography |
| — | — | — | 67.0 before chromatography<br>72.7 after chromatography |

* Upjohn.
† Blood-buffer medium.
‡ Two hour incubation.

corticosterone were added at the start of the incubation. The glycogenic activity is significantly higher than that of the controls and is thus in agreement with the data of Hayano, Dorfman and Prins (1949). When other derivatives of desoxycorticosterone such as the glucoside and the aqueous preparation of desoxycorticosterone acetate[3] were employed, similar results were obtained.

When one half of the volume of buffer solution was replaced by fresh beef blood there was a definite increase in the amount of glycogenic material (Table 1, column 3). The effect of the inclusion of blood is illustrated by the rise of glycogenic activity from an average of 6.0 ml. (equivalents of standard adrenal extract) for incubations without blood (column 2) to an average of 15.9 ml. equivalents for those containing blood (column 3). Incubations in which ascorbic acid, riboflavin and glutathione respectively were added to the incubation mixtures did not show any increase in glycogen titers above those of the respective controls.

The rate of conversion of desoxycorticosterone to glycogenically active material is shown in Figure 1. It is evident that glycogenic material is very rapidly formed, reaching a maximum in one hour, the four hour value not being significantly higher than the one hour value.

A question which arose in the early phases of this study concerned the

possibility of synergism of some non-steroid material with the very low glycogenic activity of desoxycorticosterone (Sass-Kortsák, Wang and Verzár, 1949; Wang and Verzár, 1949). The relative glycogenic potency of desoxycorticosterone with and without added ascorbic acid was determined and was found to be unaffected by such a combination. Extracts of blank control incubations, themselves slightly glycogenic (Table I, column

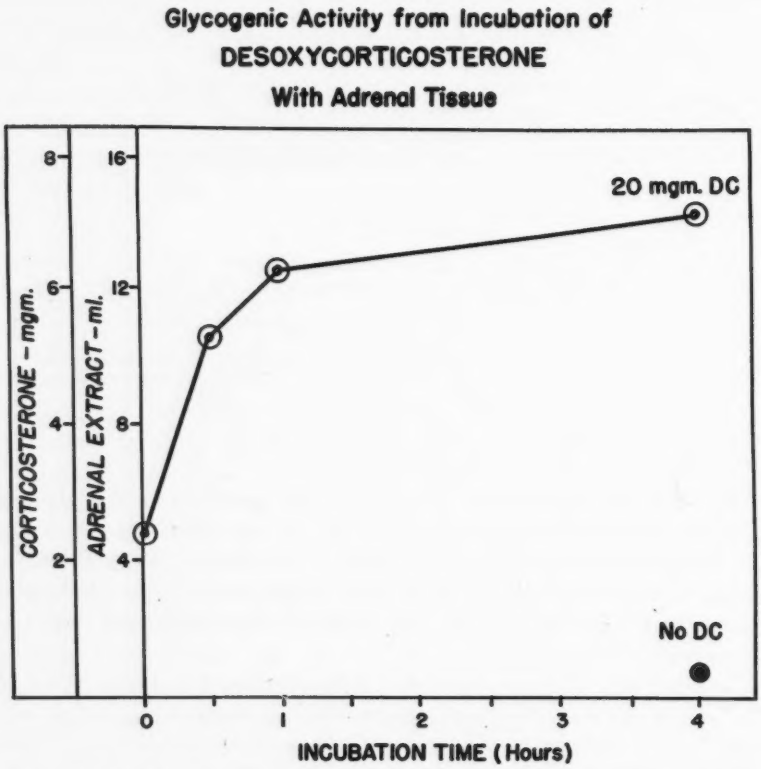


FIG. 1. Rise in glycogen titer during incubation of 20 mg. of desoxycorticosterone with 25 gm. of homogenized adrenal tissue in blood-buffer medium at 37° C.

1) were suspected of containing non-glycogenically active materials which in themselves might possibly enhance the low activity of desoxycorticosterone One experiment dealing with this point may be described, in which 20 mg. of desoxycorticosterone was added to the extract of a blank control incubation *after* the incubation was completed. The glycogen assay value of this mixture was equal to that of the same extract before the addition of desoxycorticosterone (Fig. 1, solid dot, lower right side). The conclusion that no synergising material is present or is formed during the incubation

of adrenal tissue, capable of enhancing the low glycogenic activity of desoxycorticosterone, was borne out by the chromatographic analyses.

Incubations of 17-hydroxy-11-desoxycorticosterone with adrenal tissue were carried out under the optimum conditions determined for the incubation of desoxycorticosterone, namely 30 mg. of the acetate in a homogenate of 25 g. of adrenal tissue in the blood-buffer medium. The resulting extracts possessed very high glycogenic activity when assayed before and after chromatographic purification (Table 1, column 4). In both incubations the potency levels suggest (assuming the same order of steroid conversion) the formation of a more highly glycogenic steroid than occurs on incubation of desoxycorticosterone; this is shown to be true by the chromatographic studies described below. When these glycogenic activities are corrected to an equimolecular basis (44.3 ml. equivalents of the standard adrenal extract) and compared to those of desoxycorticosterone incubations (15.6 ml. equivalents) they indicate the expected glycogenic activity ratio (3:1) between 17-hydroxycorticosterone and corticosterone.

*Chromatography:* The chromatographic examination of active extracts from the incubation of desoxycorticosterone with adrenal tissue revealed (Fig. 2) the presence of two main zones of reducing steroid material. The slowest moving spot (stippled area) in the region of $C_{21}O_5$ corticosteroids, was essentially pigmented material present before treatment of the strip with the silver reagent; this material is considered to be related to oxidized adrenalin or adrenalin-like compounds. The fastest moving spot has been identified as unchanged desoxycorticosterone (Fig. 2, strips 1 and 3). This latter spot is absent from incubation extracts where desoxycorticosterone was not added (Fig. 2, strip 2) and is present in all extracts in which the steroid was added (strips 3, 4, 5). The spot of intermediate mobility, in the anticipated region of $C_{21}O_4$ steroids, appears only in incubation extracts with enhanced glycogenic activity (strips 4, 5). It is absent from control incubation extracts where no desoxycorticosterone was added (strip 2) and is absent from those to which the steroid substrate was added but which were not permitted to incubate (strip 3) and is also absent from those with added substrate allowed to incubate but in which, for reasons unknown, glycogenic activity failed to increase. Of the three steroid zones, only the $C_{21}O_4$ zone was associated with increased glycogenic activity. Large scale chromatograms carried out on active extracts permitted the isolation of the material contained in these three zones. Biological assay of each of the areas revealed that the enhanced glycogenic activity of the incubation extract over that of the control was due to the activity found in the eluted $C_{21}O_4$ area; this eluted material accounted for about sixty-five percent of the total activity of the material chromatographed, while the fast moving (desoxycorticosterone) zone and the pigmented ($C_{21}O_5$) zone contained very slight biological activity.

Figure 3 illustrates the method of mixed chromatograms employed in determining the probable nature of the glycogenic steroid located in the $C_{21}O_4$ zone. Strip 1 shows the result of a twenty-four hour chromatogram

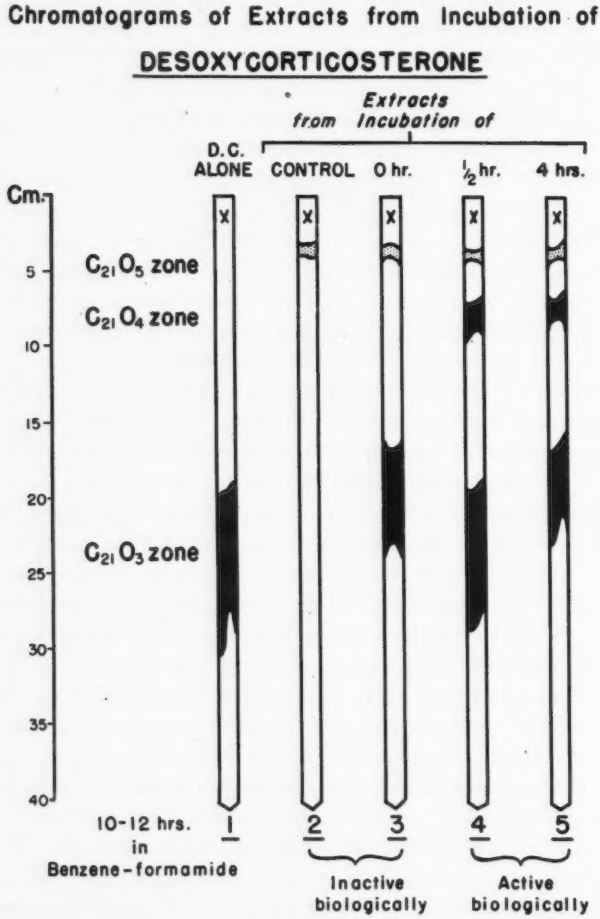


FIG. 2. Patterns resulting from chromatographic separation of approximately 1/250th of total incubation extract, or about 100 μg. total steroid. D. C. = desoxycorticosterone.

from which the fast moving desoxycorticosterone has run off the end. To the remaining strips has been added the same amount of extract as in strip 1, but to each approximately 50 μg. of corticosterone, 11-dehydrocorticosterone and 17-hydroxy-11-desoxycorticosterone respectively were added. In strip 2, the addition of corticosterone produces an enhancement of the reducing spot without separation. In strip 3, 11-dehydrocorticosterone ap-

pears as a separate distinct spot with a faster rate of mobility than the active $C_{21}O_4$ steroid. The solvent system benzene-formamide does not separate corticosterone and 17-hydroxy-11-desoxycorticosterone, but the use

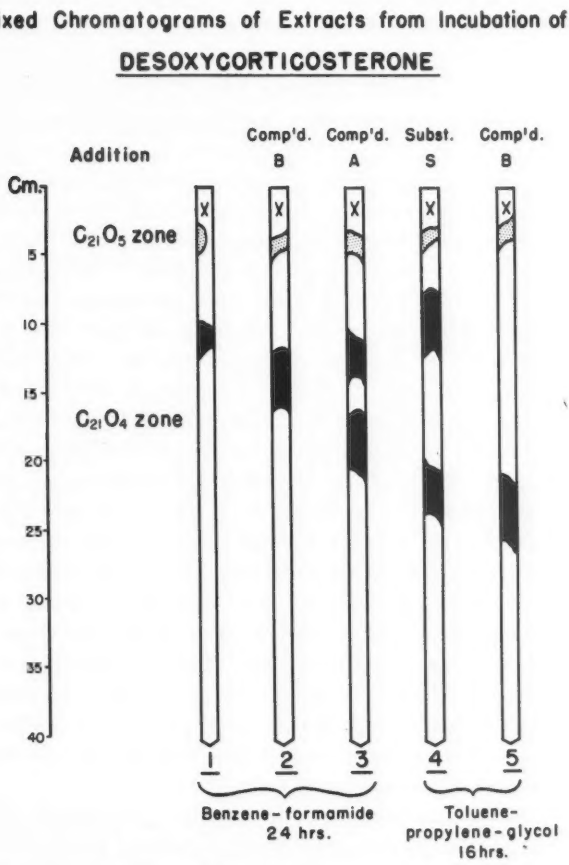


FIG. 3. Patterns resulting from chromatographic separation of about 1/250th of total incubation extract. Compound A = 11-dehydrocorticosterone: Compound B = corticosterone: Substance S = 17-hydroxy-11-desoxycorticosterone.

of toluene-propylene glycol does. This system provided a differentiation between the active $C_{21}O_4$ steroid and 17-hydroxy-11-desoxycorticosterone (strip 4) while added corticosterone (strip 5) did not separate. This evidence points to the distinct difference between the active $C_{21}O_4$ steroid and the two corticosteroids 11-dehydrocorticosterone and 17-hydroxy-11-desoxycorticosterone. The identical behavior of the active steroid with that of corticosterone in all mixed chromatograms is evidence of its prob-

able identification as corticosterone; further evidence for this is the fact that the material eluted from the $C_{21}O_4$ area of the preparative chromatograms gave a strong fluorescence with concentrated sulfuric acid indicative of an 11-hydroxyl group and possessed a high ultraviolet absorption peak at 240 m$\mu$., the region of absorption of $\alpha$, $\beta$-unsaturated ketones. It is interesting to note the complete absence of any 17-hydroxylation by the tissue homogenate which would have been indicated by the presence of 17-hydroxy-11-desoxycorticosterone or 17-hydroxycorticosterone.

Chromatographic examination of extracts of high glycogenic activity resulting from the incubation of 17-hydroxy-11-desoxycorticosterone acetate was carried out in the same way. Figure 4 illustrates the results of this study. Chromatography of the active extract and development with the silver reagent revealed two major zones and two minor zones of reducing steroid (strips 1 and 2). Three of these zones are located in the $C_{21}O_4$ area and one of the major zones in the $C_{21}O_5$ area, all spots being absent from control incubation extracts to which no steroid had been added. The pigmented area already noted above was also present. Preparative chromatograms permitted the biological assay of each of these zones. Glycogenic activity was found to be confined exclusively to the spot in the $C_{21}O_5$ zone, in which eighty-five percent of the total glycogenic activity was found. Further chromatographic examination of the three inactive $C_{21}O_4$ spots showed that the fastest moving one of these, of low concentration, was unchanged 17-hydroxy-11-desoxycorticosterone acetate; this spot was not present in extracts where incubation continued for four hours, which implies that adrenal tissue or blood is capable of hydrolysing these steroid acetates, as has already been shown by Hechter *et al.* (1949, 1950). The slowest moving $C_{21}O_4$ steroid of low concentration has not been identified; it differs in chromatographic behavior from any related steroids available for study at the time. The main $C_{21}O_4$ steroid of intermediate mobility was 17-hydroxy-11-desoxycorticosterone.

Mixed chromatograms relative to the active $C_{21}O_5$ steroid are also shown (Figure 4). Strips 1 and 2 contain equal amounts of the extract chromatographed for 16 and 72 hours respectively. Strip 3 is the same but to which approximately 50 $\mu$g. of 17-hydroxy-11-dehydrocorticosterone was added. This chromatogram shows the added known steroid as a separate distinct spot with a faster rate of movement than the active compound. Spraying of an identical chromatogram with a solution of iodine in potassium iodide (Zaffaroni, Burton and Keutman, 1950) a reagent which on filter paper produces a blue coloration with the 17-hydroxy-11-dehydrosteroid of all known glycogenic steroids, resulted in the formation of a blue spot corresponding to the added 17-hydroxy-11-dehydrocorticosterone only; chromatograms of the extract alone (strips 1 and 2) also failed to give a positive reaction in the locations of any of the reducing steroids. Strip 4 shows the

result of the addition to the usual amount of extract of about 50 μg. 17-hydroxycorticosterone before chromatography. The result was the enhancement in concentration of reducing steroid at the active $C_{21}O_5$ spot without any separation. Repetition of these mixed chromatograms in the solvent system toluene propylene glycol gave identical results, thus estab-

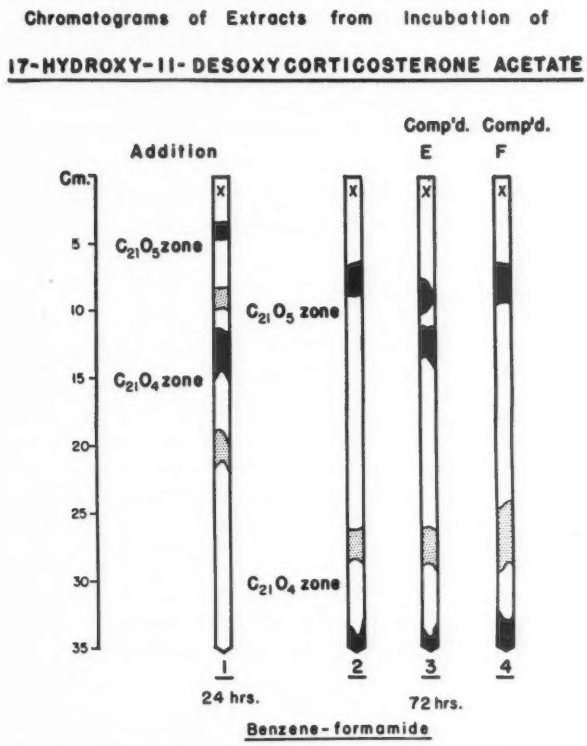


FIG. 4. Patterns resulting from chromatographic separation of approximately 1/250th of total incubation extract. Compound E = 17-hydroxy-11-dehydrocorticosterone: Compound F = 17-hydroxycorticosterone.

lishing the probable identity of the active $C_{21}O_5$ steroid as 17-hydroxycorticosterone. This is further sustained by the properties of the material eluted from this zone in the preparative chromatograms. Ultraviolet absorption measurements showed the presence of an $\alpha, \beta$-unsaturated ketone, and strong fluorescence with concentrated sulfuric acid indicated the presence of an 11-hydroxyl group.[4]

[4] Note added in press. There has recently appeared (Science *112:* 507, 1950) a communication by McGinty *et al.* on the incubation of 17-hydroxy-11-desoxycorticosterone with adrenal tissue in which the results are in agreement with those described here.

SUMMARY

The finding of the formation of glycogenic material on incubation of desoxycorticosterone with homogenates of adrenal tissue has been confirmed.

The addition of homologous blood to these homogenates greatly enhances the amount of glycogenic material formed.

Increased biological activity is associated with the appearance of a glycogenic steroid, identified by means of paper chromatography as corticosterone.

Similar incubations of 17-hydroxy-11-desoxycorticosterone (acetate) with adrenal tissue results in the formation of high glycogenic activity.

Chromatographic studies show that in these latter incubations enhanced activity is associated with the formation of a glycogenic steroid identified as 17-hydroxycorticosterone.

*Acknowledgements*

We wish to thank the following for having supplied many of the crystalline corticosteroids and other material employed in this study: Dr. M. H. Kuizenga of the Upjohn Co. for generous amounts of an aqueous adrenal extract used as standard for the biological assays; Dr. Edward Henderson of the Schering Corp., for desoxycorticosterone; Dr. Kenneth W. Thompson of Organon Inc. and Dr. B. J. Brent for the desoxycorticosterone glucoside and the aqueous preparation of desoxycorticosterone acetate; Mr. Adrian D. Joyce of the Glidden Co. for 17-hydroxy-11-desoxycorticosterone acetate; Prof. Gregory Pincus and Dr. Roger Jeanloz for corticosterone; Dr. Ralph Dorfman for 17-hydroxycorticosterone and Dr. Harold L. Mason for 11-dehydrocorticosterone.

We also acknowledge the assistance of Mrs. Ann Olsen and Miss Joanna Jones with the biological assays and Miss Ruth Joseph with the chromatographic studies.

The kindness of Dr. Alejandro Zaffaroni is acknowledged for having made available before publication the manuscript of the paper on chromatography of corticosteroids.

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# THE EFFECT OF HYPOPHYSECTOMY ON THE TOTAL CIRCULATING RED CELL VOLUME OF THE RAT[1]

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STUDIES on the influence of the pituitary on hematopiesis have resulted in general agreement that following hypophysectomy there is a 30% anemia as judged by a reduction in the hemoglobin concentration, red blood cell count, and hematocrit (reviewed by Arvy, Gabe and Stutinsky, 1948). The determination of the concentration of red blood cells and hemoglobin in the peripheral blood gives no real indication of the total circulating red cell volume because of the possibility of the influence of changes in the blood volume. This study was designed to determine the changes in the total circulating red cell volume following hypophysectomy in the rat.

## METHODS

Blood volume determinations were done by the red cell dilution method using $P^{32}$-labeled erythrocytes (Berlin *et al.*, 1949). Female rats of the Long-Evans strain were hypophysectomized at 26–28 days and the blood volume determinations were done on groups of animals prior to hypophysectomy and at various intervals up to 258 days following the operation (Table 1 and Table 2 and Figure 1). All animals were maintained on a standard laboratory diet (Nelson and Evans, 1947) ad libitum with a supplement of fresh lettuce twice weekly, and were kept in a constant temperature room throughout the experiment. Hypophysectomy was performed through the parapharyngeal approach under ether anesthesia. Completeness of hypophysectomy was determined at autopsy by examination of the pituitary site with the dissecting microscope.

## RESULTS

Figure 1 shows that the total circulating red cell volume decreased from an initial value of 2.87 cc. per 100 g. body weight at 28 days of age to 1.18 cc. per 100 g. body weight 258 days after hypophysectomy. This represents a 59% decrease in the total circulating red cell volume per 100 g. body weight. Figure 1 also shows that the total circulating red cell volume of normal animals decreased from 2.87 cc. per 100 g. body weight at 28

Received for publication July 5, 1950.

[1] This work was supported in part by the Atomic Energy Commission (Donner Laboratory) and the U. S. Public Health Service Grant RG-409 (C2) (Institute of Experimental Biology).

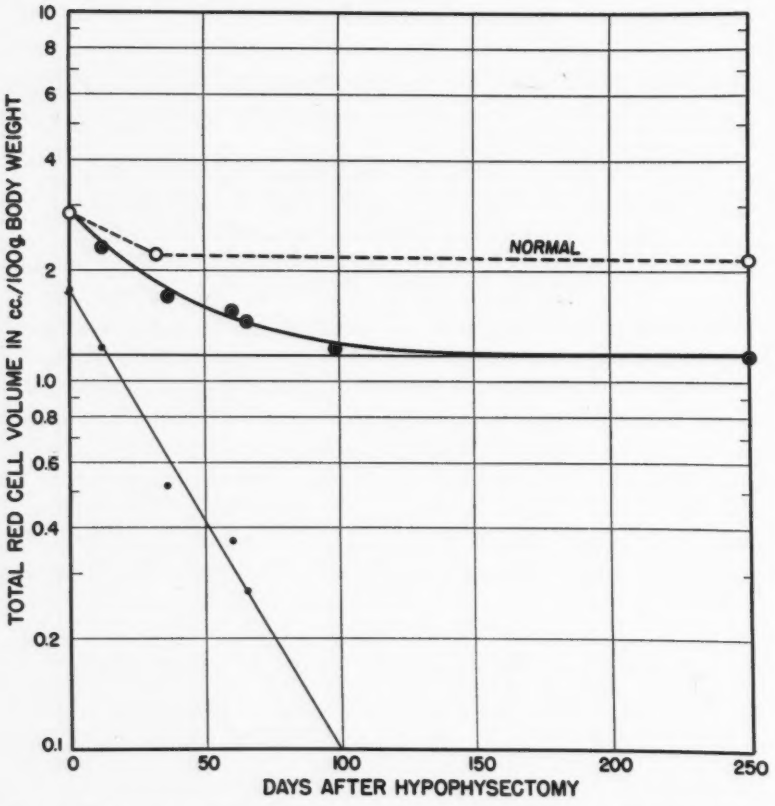


FIG. 1. The total red cell volume of the normal and hypophysectomized rat. O-Normal. ●-Hypophysectomized rat. The straight line with small dots represents the rate of destruction of the excess total red cell volume. This is obtained by subtracting the steady state value from the points on the line for the total red cell volume of the hypophysectomized rat.

days of age to 2.16 cc. per 100 g. of body weight in the adult, which represents a decrement of 25%. By the 258th postoperative day the hypophysectomized animals had established a new level of total circulating red cell volume which was 45% lower than normal rats of the same age (adult). Thus the 30% decrease in red blood cell count, hematocrit, and hemoglobin (Arvy, Gabe and Stutinsky, 1948) does not give an indication of the change in the total circulating red cell volume following hypophysectomy.

The fall in total red cell volume (Fig. 1) may be approximated by a single exponential function for which the rate constant is 0.027 of the remaining

excess red cell volume per day (excess above the steady state volume after 258 days). Table 3 shows that the mean corpuscular volume and mean corpuscular hemoglobin of the 258 day animals were not markedly different from that of normal adult rats.

TABLE 1. HEMATOLOGICAL DATA ON HYPOPHYSECTOMIZED RATS AT VARIOUS POST-OPERATIVE AGES

| Post-operative age Days | Blood volume, cc./100 g. body weight | Weight, g. | Hematocrit | TRCV* cc./100 g. body weight |
|---|---|---|---|---|
| 14 | 4.70 | 77 | 49.5 | 2.32 |
| | 4.89 | 82 | 44.0 | 2.15 |
| | 5.38 | 78 | 54.0 | 2.42 |
| Average | 4.99 | 79 | 46.2 | 2.29 |
| 37 | 4.73 | 74 | 36.0 | 1.70 |
| | 4.71 | 70 | 40.0 | 1.88 |
| | 4.10 | 73 | 38.0 | 1.57 |
| | 4.30 | 72 | 35.0 | 1.50 |
| | 4.45 | 74 | 42.0 | 1.87 |
| Average | 4.46 | 73 | 38.0 | 1.70 |
| 60 | 4.78 | 70 | 32.0 | 1.53 |
| | 3.85 | 87 | 41.0 | 1.58 |
| | 4.70 | 95 | 33.0 | 1.55 |
| Average | 4.44 | 84 | 35.3 | 1.55 |
| 66 | 3.44 | 93 | 35.0 | 1.20 |
| | 4.16 | 89 | 39.8 | 1.61 |
| | 3.88 | 93 | 38.0 | 1.47 |
| | 4.50 | 78 | 34.0 | 1.53 |
| Average | 3.99 | 88 | 36.5 | 1.45 |
| 98 | 4.40 | 95 | 26.0 | 1.15 |
| | 3.80 | 85 | 28.0 | 1.06 |
| | 3.90 | 79 | 33.0 | 1.28 |
| | 4.10 | 106 | 34.0 | 1.39 |
| Average | 4.05 | 91 | 30.3 | 1.22 |
| 258 | 3.25 | 163 | 38.0 | 1.24 |
| | 3.33 | 159 | 31.0 | 1.02 |
| | 4.24 | 106 | 29.0 | 1.18 |
| | 3.98 | 133 | 32.0 | 1.28 |
| Average | 3.70 | 140 | 32.5 | 1.18 |

* Total circulating red cell volume.

## DISCUSSION

Explicitly this experiment only provides a picture of the gross changes in total red blood cell volume following hypophysectomy. However, the characteristics of the observed changes in total red blood cell volume suggest a physical model that describes the probable influence of the rates of

production and destruction of red blood cells on the total circulating red blood cell volume.

When the total red blood cell volume remains constant the rates of production and destruction are, of course, equal although not necessarily constant. If the equality is disturbed, a steady state no longer exists and the

TABLE 2. THE BLOOD VOLUME AND CIRCULATING RED CELL VOLUME OF NORMAL 28 DAY OLD, 60 DAY OLD, AND ADULT RATS

| Age | Blood volume, cc./100 g. body weight | Weight, g. | Hematocrit | TRCV† cc./100 g. body weight |
|---|---|---|---|---|
| 28 days | 6.0 | 73 | 45.0 | 2.71 |
| | 6.1 | 72 | 46.0 | 3.00 |
| | 6.7 | 61 | 45.0 | 3.02 |
| | 6.3 | 67 | 44.0 | 2.76 |
| Average | 6.3 | 68 | 45.0 | 2.87 |
| 60 days | 4.7 | 186 | 47.5 | 2.22 |
| | 4.9 | 180 | 44.0 | 2.16 |
| Average | 4.81 | 183 | 45.8 | 2.19 |
| Adult* Average | 4.59 | 231 | 45.8 | 2.16 |

* Berlin, Huff, Van Dyke and Hennessy (1949).
† Total circulating red cell volume.

TABLE 3. HEMATOLOGICAL DATA ON NORMAL AND HYPOPHYSECTOMIZED RATS

| Type of animal | Hgb., g./100 cc. | R.B.C. $\times 10^6$ mm.$^3$ | Hematocrit | M.C.V.* | M.C.H.† |
|---|---|---|---|---|---|
| Normal adult | 16.9 | 8.93 | 47.0 | 52.5 | 18.8 |
| | 16.2 | 8.51 | 46.5 | 54.3 | 19.0 |
| | 16.3 | 8.20 | 47.0 | 55.0 | 19.9 |
| | 16.4 | 8.50 | 45.0 | 51.9 | 19.3 |
| | 15.5 | 8.63 | 44.0 | 51.0 | 18.0 |
| | 17.0 | 9.78 | 48.5 | 49.6 | 17.4 |
| | 17.5 | 8.94 | 51.0 | 56.6 | 19.6 |
| | 16.1 | 9.24 | 45.0 | 48.5 | 17.5 |
| | 17.8 | 9.46 | 46.5 | 49.2 | 18.8 |
| Average | 16.6 | 8.99 | 46.7 | 52.1 | 18.7 |
| Hypophysectomized 258 days Postoperative | 14.0 | 7.6 | 38.0 | 50.0 | 18.4 |
| | 12.0 | 7.2 | 31.0 | 44.0 | 16.7 |
| | 10.0 | 6.7 | 29.0 | 43.0 | 14.8 |
| | 11.0 | 6.1 | 32.0 | 52.0 | 17.9 |
| Average | 11.8 | 7.2 | 32.5 | 47.2 | 16.9 |

* Mean Cell Volume (in cubic microns).
† Mean Cell Hemoglobin (in micromicrograms).

total red blood cell volume then tends to readjust to a new steady state level at which the rates are again equal. Following hypophysectomy either the rate of production is diminished or the rate of destruction increased, or possibly both are affected. This was reflected in the experiment described here by a logarithmic decrease in total red blood cell volume to 45% of the initial volume after correction for the concurrent decrease with age observed in the normal animals. Although several analytical expressions may

be postulated to describe this effect, the simplest formulation, in the absence of a more detailed understanding of the mechanisms involved, may be found in assuming that one or both rates are differently affected and in particular, that the rate of destruction is, to a first approximation, proportional to the volume of red blood cells present. Following hypophysectomy the normal total red cell volume was no longer maintained and the excess volume of red cells appeared to be removed at a rate described by the equation

$$\frac{dV}{dt}=r(t)-aV \quad \text{cc. per 100 g. body weight per day} \qquad \text{I}$$

in which $(V)$ is the volume of red cells. The rate of production $[r(t)]$ is assumed to be a "step" function, i.e., before hypophysectomy it had the constant value $(r_1)$ cc. per 100 g. per day; afterward it had the constant value $(r_2)$ cc. per 100 g. per day. Similarly, for generality, the rate constant $(a)$ describing the rate of destruction may also be altered from $(a_1)\%$ per day. Although $(r)$ and $(a)$ probably are not changed instantaneously, it is necessary to assume only that the time in which $(r)$ and $(a)$ are altered is very small compared to $(1/a_2)$ in order to make the change essentially stepwise. The solution of equation I is then

$$V=e^{-at}\int re^{at}dt+Ce^{-at} \qquad \text{II}$$

where $(C)$ is the constant of integration. For simplicity, time is measured from hypophysectomy $(t=0)$ at which time the total red blood cell volume is $(V_0)$. Following hypophysectomy the volume at time $(t)$ is found, on performing the integration in equation II, to be

$$V_{(t)}=\frac{r_2}{a_2}+\left(V_0-\frac{r_2}{a_2}\right)e^{-a_2t} \quad \text{cc. per 100 g. body weight} \qquad \text{III}$$

The quantity $(r_2/a_2)$ represents the steady state total red cell volume after a very long time $[t\gg(1/a_2)]$; $[V_0-(r_2/a_2)]$ represents the excess total red cell volume after hypophysectomy and $(a_2)$ the rate constant for removal of this excess total red cell volume.

It should be noted that the quantity $(1/a_2)$ obtained from the experimental curve cannot be interpreted as the mean life of the red blood cell. It is rather the mean of the residual lives of the red blood cells of all ages. Although some relation between $(1/a_2)$ and the red blood cell mean life must exist, it is impossible without further investigation into the statistical distribution of actual cell lives to establish this relationship with any certainty. At the present time it can only be stated that the cell mean life must be greater than $1/a_2$ or 37 days.

It can be seen from equation III that a value for the circulating red cell volume in the hypophysectomized animals, 45% below that for the normal controls, can result only from (1) a 45% reduction in the rate of red cell production as compared to the normal or (2) a 45% more rapid removal of the red blood cells from the circulation with the same rate of production that occurs in the normal or (3) some combination of these two factors. The evidence supporting each of these possibilities has been discussed by Vollmer *et al.* (1939); however, the evidence is not conclusive. In favor of a decreased capacity to produce red blood cells at a normal rate are hypoplasia of the bone marrow (Overbeek and Querido, 1938), a decrease in reticulocytes in the peripheral blood (Overbeek, 1936), the inability of the hypophysectomized animals to respond to decreased oxygen tension with increased red cell production (Stewart, Greep and Meyer, 1935), and the inability to demonstrate a difference in fragility of red blood cells in the hypophysectomized animal (Overbeek and Querido, 1938). The hemosiderosis of the tissues and especially of the spleen of hypophysectomized animals (Arvy, Gabe and Stutinsky, 1948), the possible influence of hypophysectomy on saponin hemolysis as discussed by Ponder (1939), and the response to phenylhydrazine and bleeding as discussed by Querido and Overbeek (1938), are given as evidence of a more rapid breakdown of red blood cells after hypophysectomy. However, the approach to a more exact understanding of the relationships of rate of red blood cell production and destruction to the decrease in circulating red cell volume following hypophysectomy will probably be through iron turnover studies and red blood cell life determinations in such animals.

It is of interest to note that the blood volume and total red cell volume of the 28 day old normal rat were greater than that of the 60 day old normal rat. The total red cell volume of the 28 day old normal rat was 2.87 cc. per 100 g. body weight, while at 60 days of age it was 2.19 c. per 100 g. body weight (Table 2).

## CONCLUSIONS

1. Following hypophysectomy there was a decrease in the total red cell volume from 2.87 cc. per 100 g. body weight at 28 days of age to 1.18 cc. per 100 g. body weight at 258 days after hypophysectomy. The latter was 45% of the value for normal animals of a comparable age. This decrease appeared to be exponential in nature and had a rate constant of 0.027 per day.

1. The factors which may be operative in producing a 45% decrease in the total red cell volume after hypophysectomy, as compared to the normal have been discussed.

3. Young rats (28 days of age) had a larger blood volume and total red

cell volume in proportion to body weight than did the adult rats (60 days of age).

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# EFFECT OF INSULIN INJECTIONS REPEATED AT BRIEF INTERVALS*

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IN RECENT articles we presented experimental evidence to the effect that insulin action is self-inhibiting and even self-terminating, as soon as it produces hypoglycemia. This phenomenon was ascribed to the mobilization by hypoglycemia of factors (in the main: hormones of the pituitary-adrenal system) which counteract insulin (Somogyi, 1948a and b). We have demonstrated, furthermore, that this defensive mechanism goes promptly into action as soon as the blood sugar falls but a few milligrams below the normal fasting level (Somogyi, 1948a) i.e., long before the onset of subjective symptoms of hypoglycemia. From this fact it follows that when insulin is injected in the postabsorptive state, the ensuing changes in the blood sugar do not truly represent the action of the injected insulin supply. This is so, because the blood sugar declines below the fasting level immediately after injection, and this change, in turn, promptly mobilizes the insulin antagonistic factors; the changes in the glycemic level, then, are contingent on a dynamic balance between the action of insulin in one direction and of its antagonists in the opposite direction, and the course of the blood sugar curve reflects the quantitative relationship between insulin and its antagonists rather than the potential action of the supply of active insulin. The experimental evidence previously presented points to the validity of this concept; the studies submitted in this paper lend, we believe, further support to it.

## EXPERIMENTAL

Our experiments were planned as a counterpart of the procedure involved in the so-called Staub-effect. As it is commonly known, if two identical doses of glucose are administered to healthy persons at certain intervals of time, the second dose produces a lesser rise in the blood sugar than the first. The accepted explanation is that the hyperglycemia produced by the first dose of glucose stimulates the islands of Langerhans, so that the second dose meets with an increased insulin supply, which accelerates its assimilation (transfer from blood into tissue cells). By the same token, we assumed, consecutive injections of insulin should produce a similar effect

Received for publication July 13, 1950.

* This work was aided by the David May-Florence G. May Fund.

in the reverse; that is to say, if the hypoglycemia produced by a dose of injected insulin mobilizes antagonistic hormones, then a second dose, injected after a certain interval of time, should be less effective in depressing the glycemic level than the preceding one. This actually proved to be the case.

In the graph, Fig. 1, is presented an example of experiments showing this phenomenon. In this experiment a healthy young man was injected intravenously with 3 units of insulin in the postabsorptive state. As may

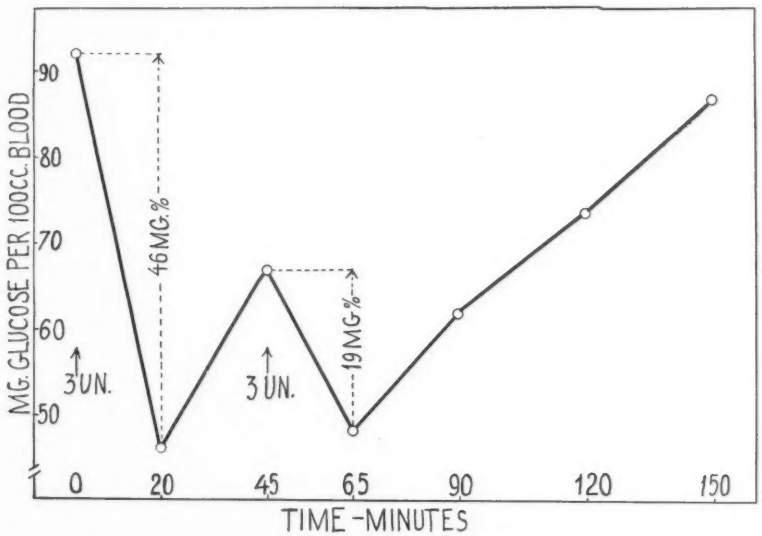


FIG. 1. Shows changes in the blood sugar level when two consecutive doses of insulin are injected intravenously, the second dose being given while hypoglycemia caused by the first dose still persists. Hypoglycemia inhibits the action of the second dose. Curve is mirror image of "Staub-effect" curve, which is obtained by administration of two consecutive doses of glucose, provided that hyperglycemia still persists when the second dose is given.

be seen, the glycemic level was depressed by 46 mg. per cent in 20 minutes (this is the time which, in our experience, is usually required to reach the lowest point with a small dose of intravenous insulin). A second 3-unit dose was then injected 45 minutes after the first, *when hypoglycemia had still obtained.* This time, as the graph shows, the blood sugar had fallen only 19 mg. per cent. The weakening of insulin action at this point is further accentuated by the fact that when the second dose was administered, approximately 2 active units of the first dose, according to Drury and Greely (1939), were still in circulation, so that the second injection had presumably

augmented the active insulin supply to about 5 units. It may be noted that the blood sugar curve obtained in this test represents a perfect mirror-image of a curve obtained after two successive glucose feedings, provided that the second dose is fed while the hyperglycemia produced by the first still persists.

In a control test we again gave the subject two consecutive insulin injections of 3 units each, as in the preceding test, but with the difference that the time interval between the two injections was 80 instead of 45 minutes. During this prolonged interval, as we have observed in past

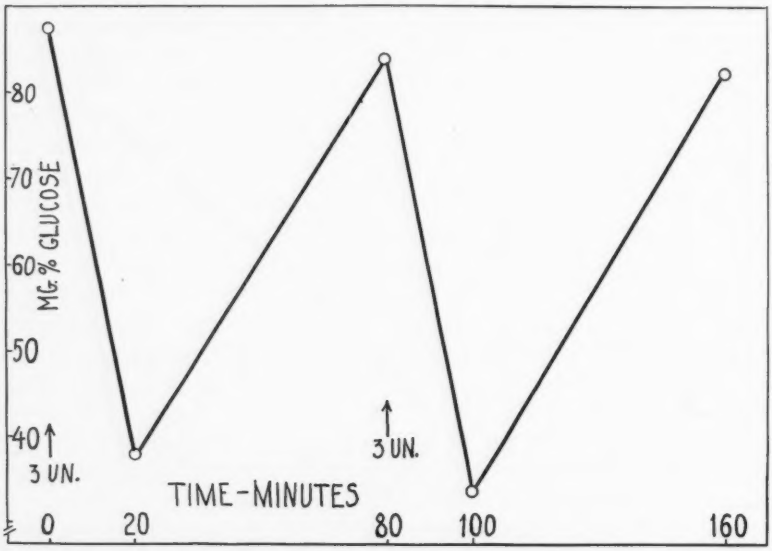


FIG. 2. Changes in blood sugar level after injection of two consecutive doses of insulin, if the blood sugar is allowed to return to its normal level before the second dose is injected. No inhibition of the action of the second dose is in evidence.

studies, the glycemic level can return from its lowest point to its normal level. It was assumed that to the same degree as the blood sugar rises, the stimulus of hypoglycemia upon the pituitary-adrenal mechanism diminishes, and nearly subsides by the time the normal glycemic level is restored. If this assumption is correct, then the second dose of insulin, injected at this point, meets with only negligible resistance, and should act about as effectively as the first. As Fig. 2 shows, the experiment has fully borne out our assumption. It is to be remembered that some of the first insulin dose (roughly about one-half of it) was presumably still active 80 minutes after injection and, hence, when the second dose was injected, the insulin *supply*

was greater than at the first injection. This excess, it appears, just sufficed to balance the residual insulin antagonists still present, and thus insulin *action* was approximately of the same extent as after the first injection. This curve is again the mirror-image of one obtained with two consecutive glucose feedings, provided that the second dose is fed after the hyperglycemia produced by the first has already subsided.

In another experiment five consecutive insulin doses of 2.5 units each were injected, at 35-minute intervals, to a healthy young man weighing 64

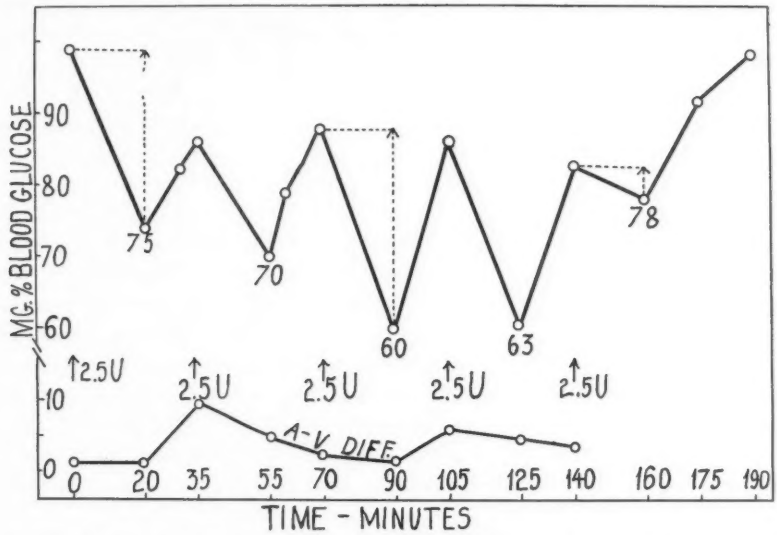


FIG. 3. The effect of five consecutive injections of small doses of insulin (2.5 units each) to healthy adult man. Inhibition by hypoglycemia is slight until the cumulative insulin supply derived from 3 successive injections had caused a "critical blood sugar level," about 60 mg. per cent, which excited the pituitary-adrenal mechanism sufficiently to inhibit insulin action. In consequence, inhibition was almost complete after the 5th injection, despite the fact that the supply of active insulin at that time was greater than during any of the preceding intervals. The activity of insulin antagonistic factors is also reflected in the narrowness of A–V differences.

kilos. The result is presented in Fig. 3. The first injection, as may be seen, lowered the blood sugar only to 75 mg. per cent. The second injection exerted less effect by far, but after the third dose—in seeming conflict with our assumption—the hypoglycemia deepened substantially, reaching the low point of 60 mg. per cent. From then on, however, the effect of the subsequent injections weakened, so that the 5th injection lowered the glycemic level only by 4.5 mg. per cent (as against a depression of 28 mg. per cent elicited by the 3rd insulin dose).

We interpret the course of the blood sugar curve in Fig. 3 as follows: The first two 2.5 unit doses caused but slight degrees of hypoglycemia—the lowest point was 70 mg. percent—hence the stimulus exerted on the insulin antagonistic mechanism was correspondingly slight. In consequence, the 3rd insulin dose, added to the residual active supply of the preceding two doses, was capable of further depressing the blood sugar to 60 mg. per cent. This hypoglycemic level then sufficed to intensify the insulin antagonistic response to a degree which prevented the next insulin dose from deepening the hypoglycemic level any farther. Yet the hypoglycemic swing following this, the 4th, injection still was great enough to mobilize additional antagonistic forces, and it was the result of this added inhibitory reaction that the 5th injection had become almost entirely ineffective in lowering the blood sugar; that is to say, that its action was virtually overpowered and nullified by the force of the accumulated antagonistic factors.

For a correct evaluation of the magnitude of the insulin antagonistic action it must be taken into consideration that when the 5th injection was almost fully inhibited in its action, it actually was not 2.5 units of insulin that was stalemated by the antagonistic factors, but about 8 units, since approximately 5.5 of the previously injected 10 units were still present in active state (as calculated according to Drury and Greely, 1939). This shows that in the course of two hours the gradually deepening hypoglycemic states elicited an accumulation of insulin antagonists in such a quantity, that 8 units of insulin exerted incomparably less action than the 2.5 units which were injected at the beginning of the test, i.e., when the pituitary-adrenal mechanism had not yet been alerted for defense against hypoglycemia.

Time and again in the course of our studies we have gained the impression that hypoglycemic levels which are not below 70 mg. per cent, elicit only mild responses from the insulin antagonistic mechanism. This may be seen in Fig. 3. It is evident from this curve that the hypoglycemia of 75 mg. per cent, which was produced by the first 2.5 units of insulin, failed to mobilize antagonistic factors in a quantity to prevent the progressively increasing hypoglycemic action of three consecutive doses of insulin. Only when the third dose finally lowered the blood sugar to 60 mg. per cent, were the antagonistic factors sufficiently reinforced to break the tide of insulin action (i.e., to prevent further intensification of hypoglycemia).

Thus it appeared that in nearly all of our subjects the blood sugar had to fall to about 60 mg. per cent in order to mobilize a fairly energetic defense force of insulin antagonistic factors. The validity of this assumption was probed by an experiment on the same subject who had served in the preceding experiment. On this occasion he received 4 units of insulin as the first dose and, as previously, 2.5 units 35 minutes later. As may be seen in

Fig. 4, the initial 4 units depressed the blood sugar to 58 mg. per cent, i.e., to the supposed "critical level." As a direct consequence, it appears, the next insulin dose of 2.5 units virtually failed to depress the glycemic level any farther, despite the fact that at the time (35 minutes after the first injection) a total insulin supply of no less than 5 units were available for action.

It may be more than a coincidence that this finding is in close agreement with the observation of Cannon, McIver and Bliss (1924), who regarded

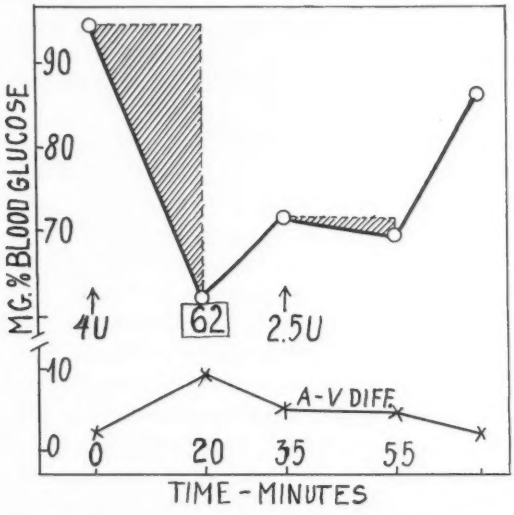


FIG. 4. This graph shows that when the first of two consecutive insulin doses is just large enough to depress the blood sugar to the "critical level," the action of the second dose is virtually blotted out by the mobilized insulin antagonistic factors. Comparison of the shaded areas shows that the action of the second dose was virtually negligible. While the first dose still caused a slight increase in A-V difference, the second dose failed to produce such effect, indicating the interference of insulin antagonistic factors.

about 80 mg. per cent as the critical blood sugar level which elicits a *measurable* increase of epinephrine secretion in rabbits. In Cannon's work blood sugar was determined by the Folin-Wu method which yields values on an average 20 mg. per cent higher than the actual glucose content of the blood as determined by our method, so that their 80 mg. per cent corresponds to a true sugar value of 60 mg. per cent.

It was shown in our previous studies that the insulin antagonistic factors which are mobilized by hypoglycemia, depress the rate of peripheral glucose assimilation, as measured by changes in arteriovenous (A–V) differences (Somogyi, 1948a and b). This reaction was in evidence also in the

present experiments. In Figs. 3 and 4, the curves in the lower part of the graphs show that A-V differences were increased only after the first dose of insulin, and then to no more than 10 mg. per cent. Under the influence of continued hypoglycemic levels, however, the A-V differences receded within the limits of the normal postabsorptive range in spite of the gradual accumulation of the active insulin supply. In moderately hyperglycemic subjects (mild diabetic persons) on the other hand, the A-V difference increases to 30 and even 40 mg. per cent in 20 minutes after the intravenous injection of from 3 to 5 units of insulin in the postabsorptive state (Somogyi 1948b). This is so because in these subjects the blood sugar can fall a long way without hitting hypoglycemic levels, hence the pituitary-adrenal system is not affected and insulin action can proceed uninhibited.

## SUMMARY

Consecutive intravenous injections of small doses of insulin to healthy persons produce changes in the blood sugar level which present the mirror-image of the changes produced by consecutive administrations of glucose. The phenomenon following repeated glucose feeding (Staub effect) is ascribed to stimulation of insulin secretion by hyperglycemia, with the consequence that the mobilized insulin holds down the hyperglycemic effect of successive glucose doses. Insulin produces a counterpart of this picture: the initial insulin-hypoglycemia stimulates the pituitary-adrenal mechanism to increased mobilization of insulin-antagonistic hormones, with the consequence that successive insulin doses exert considerably less hypoglycemic effect than the first. As a matter of fact, insulin action may be completely cancelled by the antagonistic factors which accumulate when hypoglycemia is allowed to persist for some time.

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# THE TURNOVER OF BODY FAT IN OBESITY RESULTING FROM HYPOTHALAMIC INJURY STUDIED WITH THE AID OF DEUTERIUM[1]

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THE depot fat of the normal laboratory animal has been shown to participate in the "dynamic steady state" in that its molecules are known to be replaced by new molecules at relatively rapid rates (Schoenheimer 1942). In the congenitally obese mouse, in contrast to the normal mouse, it was found that turnover of body fat was extremely slow (Salcedo and Stetten 1943). It was considered desirable to determine whether other forms of obesity were associated with the same slow turnover.

The obesity which follows surgical injury to the hypothalamus has been shown to be associated with a marked increase in food intake rather than with a diminution in the overall metabolic rate (Brobeck 1946; Stevenson 1949; Stevenson, Welt and Orloff in press; Brobeck, Tepperman and Long 1943). The present experiments were designed to measure the rate of turnover of the fatty acids of various body fats and to ascertain, if possible, whether this obesity resulted predominantly from excessively rapid deposition of fat or from abnormally slow mobilization. To this end rats were subjected to hypothalamic injury, allowed to become obese, and then, for a period of time, were fed a diet containing deuterium-labeled fatty acids. They were then placed on a fat-poor diet, killed at specified intervals and the deuterium content of carcass, mesenteric and liver fatty acids determined. The turnover of fat in various sites could thus be estimated from the experimentally determined rate at which isotopic body fat was being replaced by nonisotopic fat.

Received for publication July 19, 1950.

[1] The work at Yale University was aided by a grant from the Life Insurance Medical Research Fund.

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## EXPERIMENTAL

Tristearin labeled in the fatty acids with deuterium was prepared by the catalytic reduction of commerical linseed oil in an atmosphere of pure $D_2$ gas. Linseed oil was dissolved in ethyl acetate, platinum oxide (Adams, Voorhees and Shriner 1932) added and the mixture stirred magnetically at room temperature under $D_2$ at slightly less than 1 atmosphere pressure. Deuterium was generated by the electrolysis of 99.8% $D_2O$. Stirring was continued until no further uptake of gas could be detected. After removal of the catalyst by filtration, the triglyceride was recovered by evaporation of the solvent. In order to enhance intestinal absorption of this product (m.p. 54–58°), it was mixed with an equal weight of Wesson oil prior to incorporation into the diet

Rats of the Sprague-Dawley strain were used. At the time of operation their mean weight was 263 gm. Under Evipal anesthesia, bilateral electrolytic lesions of the hypothalamus were produced with the Horsley-Clarke instrument. The Horsley-Clarke coordinates used were those which usually produce lesions in or near the lateral aspect of the ventromedian nuclei. The electrolytic current was 2 milliamperes for 15 seconds. During the postoperative period the animals were fed a diet *ad libitum* of calf meal 40% water 60%. Under these conditions the animals gained weight rapidly and approximately 3 months after operation their mean weight was 468 gm., 44 per cent greater than that of similarly treated unoperated controls.

Preliminary to the isotope study five such rats were placed on a basal diet containing corn starch 65%, casein 22.5%, yeast powder 6.25%, salt mixture (Osborne and Mendel 1913) 6.25%. Two drops of vitamin A and D concentrate were added to the diet every other day. Food was offered *ad libitum*. The animals consumed 30–45 gm. of this diet daily and gained weight at rates varying from 2 to 6 gm. per day. When each rat had achieved a body weight of 500–520 gm., its basal diet was replaced by a mixture comprising basal diet 189 gm., Wesson oil 10.5 gm., deuterio-tristearin 10.5 gm. This mixture contained the equivalent of 20 gm. of fatty acid, and the mean deuterium concentration of this fatty acid was 7.30 atom % excess. Each rat consumed 95–100% of this diet in seven days, after which the first rat was sacrificed (zero day). The surviving rats were returned to the original, approximately fat-free basal diet and individuals were killed after 6, 12, 18 and 24 days. During this, the experimental period, food offered *ad libitum* was consumed at the rate of 20 to 35 gm. and body weights continued to increase at 2–6 gm. per rat per day.

Rats were killed by ether anesthesia, the livers were removed, and mesenteric fat taken for separate analysis. The remaining viscera were discarded. Fatty acids were isolated by essentially identical procedures from liver, mesentery and eviscerated carcass. Each tissue was heated under reflux for 3–6 hours with 10% ethanolic KOH, and after extraction of the nonsaponifiable materials with petroleum ether, the aqueous-alcoholic solution was acidified with HCl and the fatty acids collected in petroleum ether. This solution was dried over $Na_2SO_4$, filtered, and evaporated to dryness. The deuterium analyses were performed in a mass spectrometer.

The feces of one rat were collected during the period of isotopic fat feeding. 13.7 gm. of feces were found to contain 4.18 gm. of fatty acids, after saponification.

Deuterium, 6.93 atom% excess.

## DISCUSSION

The quantities of fatty acids recovered and their deuterium concentrations are recorded in Table 1. It will be seen that each animal contained in the neighborhood of 250 gm. of fatty acids. In striking contrast to results

obtained in similar feeding experiments conducted upon normal rats and mice (Bernhard and Bullet 1943; Stetten and Grail 1943) and in agreement with observations on the congenitally obese mice (Salcedo and Stetten 1943), the concentrations of deuterium in the fatty acids of depot fat showed little tendency to diminish through time. This finding is more clearly illustrated in the graphic presentation of the data (Fig. 1). Data secured from normal rats under similar experimental circumstances re-

TABLE 1. THE QUANTITIES AND DEUTERIUM CONCENTRATIONS OF THE FATTY ACIDS OF OBESE RATS

| Days | Liver fatty acids | | Carcass fatty acids | | Mesenteric fatty acids | | Labeled fatty acids persisting in* | | | Totals |
|---|---|---|---|---|---|---|---|---|---|---|
| | Wt. gm. | D atom % | Wt. gm. | D atom % | Wt. gm. | D atom % | Liver gm. | Carcass gm. | Mesentery gm. | gm. |
| 0 | 0.63 | 0.508 | 211 | 0.146 | 42.4 | 0.100 | 0.04 | 4.22 | 0.58 | 4.84 |
| 6 | 0.98 | 0.091 | 195 | 0.139 | 62.3 | 0.106 | 0.01 | 3.72 | 0.91 | 4.64 |
| 12 | 3.29 | 0.086 | 172 | 0.131 | 49.5 | 0.102 | 0.04 | 3.08 | 0.69 | 3.81 |
| 18 | 1.78 | 0.074 | 196 | 0.151 | 57.5 | 0.111 | 0.02 | 4.06 | 0.87 | 4.95 |
| 24 | 9.44 | 0.064 | 220 | 0.116 | 70.9 | 0.076 | 0.08 | 3.50 | 0.74 | 4.32 |

* These values are calculated from the expression:

$$\frac{\text{gm. fatty acid isolated} \times \text{its deuterium concentration}}{\text{deuterium concentration of dietary fatty acid}}$$

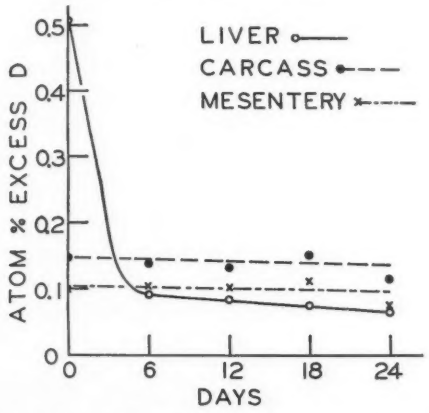


FIG. 1. Deterium concentrations of fatty acids isolated from liver, carcass and mesenteric fat of obese rats after preliminary enrichment with deuterio fat.

vealed an approximate halving of the isotope concentration for each successive nine-day interval (Bernhard and Bullet 1943).

From the experimental values the quantities of labeled dietary fatty acid which were deposited and which persisted in the tissues throughout the experimental periods have been computed (Table 1). Of the 20 gm. of fatty acids ingested, approximately 25% was recovered from the tissues analyzed. This figure may be compared with the 44% of dietary isotopic

fatty acids deposited under similar though not identical circumstances in the normal rat (Stetten and Schoenheimer 1940), and indicates that there is no excessive absorption and deposition of fat in animals subject to hypothalamic injury. The failure of the total quantities of labeled fatty acid in the tissues to show any systematic decline over a period of 24 days indicates the slowness with which mobilization is occurring and suggests the possibility that the process of fat mobilization is decreased in these animals. However, were the mobilization of fat proceeding at the normal rate—2 gm. per rat per day (Stetten and Boxer 1944)—this would amount to less than 1% per day, and even over a period of 24 days might have escaped detection. Certain it is that the rate of mobilization has not increased in proportion to the increase in quantity of depot fat, and the results suggest that in this pathological condition the depots do not participate in any highly dynamic state.

The concentrations of deuterium in the fatty acids of the mesenteric fat were consistently lower than the corresponding values in carcass fat. Whereas the significance of this finding is not entirely clear, it is apparent that at this period the deposition of fat in the mesentery is proportionately slower than in the carcass.

The deuterium concentration in the fatty acids of the liver fat fell from an initial high value to a level significantly below the concentration in the depot fats. In view of the low fat content of the diet at this time, it must be concluded that some of the liver fat arises from synthesis of isotope-poor fatty acids in the liver. Several of the livers contained quantities of fatty acids far in excess of normal, ranging from 5 to 37% of wet weight of liver.

The feces of one rat were collected during the period of labeled fatty acid feeding. The deuterium concentration of the fatty acids isolated from this material was 95% of that in the dietary fatty acids. It may be concluded that little dilution of dietary fat has occurred in the intestinal tract.

## SUMMARY

Deuterio fat has been added to the diet of rats rendered obese by hypothalamic injury, and during a subsequent period the isotope concentrations of depot and liver fat have been studied.

The *fraction* of depot fat mobilized per day has been found to be extremely small. The *quantity* of depot fat mobilized per day was likewise small but it was not possible to determine with certainty that it was less than in the normal rat.

The fatty acids of the feces during the period of feeding of isotopic fat proved to be essentially undiluted dietary fatty acids.

At the period of the experiment the deposition of dietary fatty acids in the mesenteric fat was proportionately less than in the carcass fat.

*Acknowledgements*

The authors wish to express their indebtedness to Miss Joan Willander, Mrs. Eleanor Schroeder and Mr. Frank Rennie for valuable technical assistance.

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# TESTOSTERONE PREPARATIONS WITH PROLONGED ACTIVITY[1,2]

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IT IS well known that the duration of response elicited by a given dose of hormone depends on many factors, among which are the vehicle used, introduction of chemical radicals and the physical state of the preparation (Deansley and Parkes, 1937; Sklow, 1943; Miescher, Gasche and Frey, 1944; Carlinfanti, D'Alo and Cutalo, 1949; Lens, Overbeek and Polderman, 1949; Hamburger, 1949).

The experiments reported here concern studies on the "depot action" of testosterone and tes osterone propionate. They were designed to test, under standardized conditions, the effects that the various factors mentioned above would have in modifying the response of the seminal vesicles and ventral prostate lobes to male sex hormone.

The results show that 1) an emulsion and a macrocrystalline suspension of testosterone propionate are about equally effective in eliciting a marked and prolonged stimulation of sex accessory glands, 2) testosterone and testosterone propionate adsorbed on aluminum phosphate are less effective in these respects, 3) testosterone propionate dissolved in oil is the least effective of the preparations tried and 4) addition of the propionate side-chain does not prolong the activity of testosterone when it is given adsorbed on aluminum phosphate.

## MATERIALS AND METHODS

The preparations used were: 1) macrocrystals of testosterone propionate suspended in methyl cellulose, 2) testosterone propionate emulsified according to the method of Lens *et al.* (1949), 3) testosterone propionate and testosterone (free) adsorbed on aluminum phosphate (Carlinfanti *et al.*, 1949) and 4) testosterone propionate dissolved in oil. Administration of the hormone adsorbed on aluminum phosphate or in emulsion did not require special precautions, the vial with its contents was shaken, the material quickly withdrawn by syringe and injected, using small gauge needles. For the injection of the testosterone suspension it was necessary, because of the large crystals, to use an 18 gauge needle mounted on a Luer-Lok, wide base, 2 ml. syringe. The material was injected after thorough agitation and the skin firmly pinched as the needle was withdrawn; a wound clip and celloidin were applied at the site of injection to prevent leakage.

Received for publication July 19, 1950.

[1] Aided by a grant from the Ciba Pharmaceutical Products, Inc., Summit, N. J., which made and generously supplied the hormone preparations.

[2] The authors wish to express their thanks to Dr. Robert Gaunt for aid in the preparation of the manuscript.

The method for testing the effectiveness of the various preparations was that developed by Hays and Mathieson (1945a; 1945b) and subsequently used by Wills, Rampton and Pugsley (1949). A total of 516 Sprague-Dawley and Wistar male rats, 25 to 28 days of age and weighing approximately 65 gm. were castrated and after a post-operative interval of at least two weeks a single dose of hormone amounting to 5 mg. in 0.2ml. vehicle was injected subcutaneously. Following hormone administration the animals were sacrificed in groups of 4 to 16 at 4 or 5 day intervals, the last groups being killed 40 days after injection. Immediately upon death the seminal vesicle and ventral prostate glands, with their contained seminal fluids, were dissected free of surrounding tissue, rinsed in 0.9 per cent sodium chloride, blotted, and weighted separately on a Roller-Smith balance to the nearest 0.1 mg.

## RESULTS

Figure 1 summarizes the findings in graphic form and detailed data are given in the two tables.

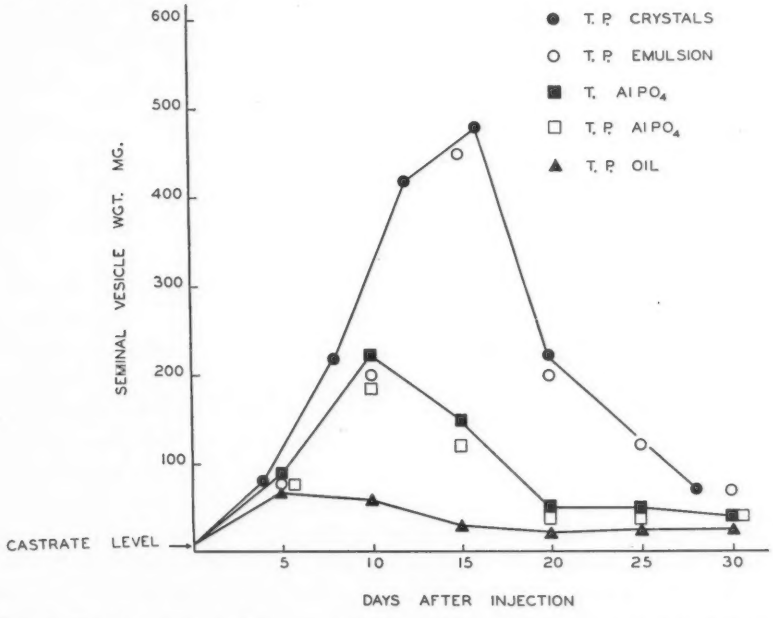


FIG. 1. Showing curves of mean seminal vesicle weights of castrate rats injected subcutaneously with one 5 mg. dose of androgen.[2]

In the cases where hormone administration was duplicated in the two strains of rats the patterns of response were very similar and probably would have been nearly identical had the animals been sacrificed at the same time intervals. Because of such similarity of response the curve for

[3] The meaning of the symbols used in the figure and tables are as follows: T = testosterone, P = propionate, Al $PO_4$ = adsorbed on aluminum phosphate.

the seminal vesicle weights of the Sprague-Dawley rats treated with the crystalline suspension was included in chart 1 with the curves for organ weights of Wistar rats that had been treated with other types of preparations.

*Effects on the seminal vesicles:* In animals receiving no treatment the seminal vesicles remained in a prepubertal condition, weighing at all intervals in the vicinity of 6 mg.

In treated rats all preparations were equally effective in stimulating the seminal vesicles during the first 5 days after injection, but thereafter differences of considerable magnitude became apparent. Between the fifth and

TABLE 1. MEAN SEMINAL VESICLE WEIGHTS OF CASTRATE RATS INJECTED SUBCUTANEOUSLY WITH ONE 5 MG. DOSE OF ANDROGEN

| | WISTAR STRAIN | | | | | |
|---|---|---|---|---|---|---|
| Number days | 5 | 10 | 15 | 20 | 25 | 30 | 40 |
| Controls No treatment | 5.5±0.2* (10)† | 6.0± 0.2 (10) | 5.4± 0.3 (10) | 5.3± 0.3 (10) | 5.8± 0.3 (10) | 6.6±0.4 (10) | 5.8±0.3 (11) |
| T.P.-oil | 73.5±2.7 (12) | 61.5±15.1 (12) | 31.2± 2.0 (12) | 22.6± 2.3 (12) | 24.2± 2.8 (10) | 24.7±1.5 (12) | 21.4±1.9 (11) |
| T.P. Al $PO_4$ | 82.3±3.1 (12) | 189.2±37.3 (12) | 118.2±32.1 (12) | 40.5± 5.3 (12) | 25.6± 3.7 (11) | 35.9±6.9 (12) | 29.5±3.3 (12) |
| T. Al $PO_4$ | 78.5±3.8 (12) | 226.3±29.1 (12) | 146.0±24.2 (12) | 46.7± 6.4 (12) | 54.4± 5.0 (12) | 34.1±4.5 (12) | 41.0±7.8 (12) |
| T.P.-emulsion | 77.6±2.8 (12) | 196.0±11.5 (12) | 457.5±48.0 (12) | 193.5±39.8 (12) | 93.2±15.9 (10) | 62.3±6.1 (12) | 50.3±2.7 (12) |
| | SPRAGUE-DAWLEY STRAIN | | | | | |
| Number days | 4 | 8 | 12 | 16 | 20 | 28 |
| T.P.-oil | 76.8±12.3 (5) | 48.0± 4.8 (4) | 33.1± 2.4 (4) | | | |
| T. Al $PO_4$ | 63.4±12.2 (11) | 238.4±17.9 (11) | 298.8±45.1 (16) | 173.3± 55.1 (9) | 65.2± 5.5 (9) | 59.6±8.9 (8) |
| T.P. Crystals | 68.0± 7.9 (5) | 219.6± 8.0 (5) | 417.3±27.0 (11) | 479.6±121.2 (5) | 228.8±45.1 (5) | 71.0±5.7 (7) |

* Standard error $\pm \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

† Number of cases.

tenth days, while there was a decline in the organ weights of the animals that had been injected with the oil solution, there was a marked increase in the seminal vesicle weight of the rats that had been injected with suspensions, emulsions and adsorbates. At the tenth day the seminal vesicles of the latter groups of animals weighed in the vicinity of 200 mg., representing more than a doubling of the weights within a 5 day period. During the interval between the tenth and fifteenth days the seminal vesicles of the emulsion- or crystal-treated animals again more than doubled in weight while the size of the glands of the adsorbate-treated rats was rapidly declining. Following the fifteenth day the seminal vesicles of the crystal- and emulsion-treated rats shrank precipitiously and by the thirtieth day were near the atrophic levels of the other groups.

*Effects on the ventral prostate:* The data in table 2 shows a pattern of response for the prostate gland which was similar to that obtained for the seminal vesicles, differing in that the prostate weights were uniformly smaller than those of the seminal vesicles. The prolonged actions of the adsorbates and the emulsion were evident but the latter was more effective than the former. These results on the prostate confirmed those obtained for the seminal vesicles and hence need not be described in detail.

TABLE 2. MEAN VENTRAL PROSTATE WEIGHTS OF CASTRATE RATS INJECTED SUBCUTANEOUSLY WITH ONE 5 MG. DOSE OF ANDROGEN

| | WISTAR STRAIN | | | | | | |
|---|---|---|---|---|---|---|---|
| Number days | 5 | 10 | 15 | 20 | 25 | 30 | 40 |
| Controls No treatment | 6.9±0.4* (10)† | 7.8± 0.6 (9) | 7.6± 0.5 (10) | 6.3± 0.3 (10) | 7.0± 0.5 (10) | 7.4±0.3 (10) | 6.5±0.4 (11) |
| T.P.-oil | 59.7±4.2 (12) | 63.2±11.7 (12) | 23.1± 1.7 (12) | 16.1± 1.7 (12) | 14.9± 1.7 (10) | 14.2±1.2 (12) | 13.5±0.7 (11) |
| T.P. Al $PO_4$ | 64.0±3.6 (12) | 121.1±14.6 (12) | 96.9± 7.2 (12) | 43.1±18.6 (12) | 15.7± 1.9 (11) | 22.4±5.0 (12) | 16.5±1.2 (12) |
| T. Al $PO_4$ | 64.9±3.6 (12) | 143.0±11.5 (12) | 122.5±23.3 (12) | 27.9± 4.1 (12) | 25.8± 3.9 (12) | 18.7±2.1 (12) | 23.8±6.1 (12) |
| T.P.-emulsion | 77.6±2.3 (12) | 159.0± 9.7 (12) | 206.5±13.8 (12) | 177.6±29.6 (12) | 73.8±23.2 (10) | 35.1±4.4 (12) | 24.4±1.7 (12) |

* Standard error $\pm \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

† Number of cases.

## DISCUSSION

Deansley and Parkes (1937) and Sklow (1943) working with crystalline preparations and Lens *et al.* (1949) and Hamburger (1949) using emulsions have previously shown that androgens are more effective when given in such forms that when they are administered in oil. Our results confirm these findings as well as those of Carlinfanti *et al.* (1949) who employed testosterone adsorbed on aluminum phosphate. Our studies differ from the above in that some of the preparations tested were new and also in that comparisons were made between the various types under standardized conditions.

The results reported here demonstrate that testosterone propionate in the form of a macrocrystalline suspension or as an emulsion stimulates the accessory sex glands of castrate rats for longer periods of time and to a greater extent than it does when it is given dissolved in oil or as an adsorbate on aluminum phosphate. Furthermore, they show that testosterone propionate in emulsion is as effective as testosterone propionate in crystalline suspension and since the former can be administered more easily than the latter it is considered to be the best injectable preparation of those tested for prolonged androgenic stimulation.

Individual variation in response to the long acting preparations is great, and greatest at the time of maximum effects. Nevertheless, the mean ef-

fects of the macrocrystals and the emulsion are significantly greater during the periods of maximum stimulation than are those of the testosterone aluminum phosphate. Using the peak seminal vesicle weights as criteria for relative effectiveness of the preparations it can be estimated that the adsorbates are about twice and the emulsion (or macro-crystals) more than five times as effective as testosterone in oil. However, it seems likely that the reason why the glands reach such a large size with some of the substances is not because of greater potency but because the hormone is absorbed and utilized more slowly and efficiently. This suggestion is further borne out by the fact that as long as the various materials cause an increase in seminal vesicle weight the mean values are approximately equal. In other words, at five days all preparations cause about the same degree of increase in weight and at 10 days all preparations having prolonged activity give weights that are not significantly different from each other.

When the stimulating actions of testosterone aluminum phosphate are compared with those of testosterone propionate aluminum phosphate it is impossible to establish significant differences, although the trend indicates that "free" testosterone is possibly a little more effective than the propionate. This lack of marked difference is of interest because of the well known fact that addition of the side chain prolongs the action of testosterone when it is given dissolved in oil.

The precise time at which the stimulating effect of the hormone ceases cannot be determined from the data given here because decreases from the maximum are probably due to gradual absorption of residual hormone and attendant slow atrophy. In other words, the decrease from the peak effect may not be due to the absence of hormone but rather to an insufficient quantity necessary to maintain the organ weights at a high level. The glands of none of the treated animals declined to the castrate level but it is possible that hormone stimulated organs acquire tissues during their growth that resist complete atrophy after hormone withdrawal. This might be an explanation for the lack of decrease in organ weights following the twenty-fifth day after injection. Apparently, the hormone effects had completely worn off some time earlier than the end of the fourth week.

The results for the ventral prostate gland show that it responds in the same pattern as does the seminal vesicle but because of ease of dissection and manipulation the latter organ is the one of choice for the routine testing of androgens.

## SUMMARY

A single subcutaneous injection of 5 mg. of testosterone propionate in the form of macrocrystals in methyl cellulose or in an emulsion stimulates the seminal vesicle and ventral prostate glands of castrate immature rats

to a greater extent and for a longer period of time than does a single injection of testosterone (free or the propionate) adsorbed on aluminum phosphate. All of the preparations tested are much longer lasting in their effects than is the commonly used solution of testosterone propionate in oil.

## ADDENDUM

Dr. C. W. Lloyd of the Medical College at Syracuse University has employed long-active preparations of testosterone in the treatment of patients and his findings are summarized below:

Five adult male humans with hypogonadism have received 40 injections of macro-crystalline suspension of testosterone propionate in methyl cellulose. Dosages varied from 50 to 100 mg. per injection. The maximal clinical response was first reached three to four days after injection and was maintained for from two to three weeks. Thereafter the effectiveness gradually decreased until no clinical response could be detected after four weeks. Although the clinical response was good, administration of the material was not practicable because of the extreme difficulty in injecting it.

Five patients received a total of 12 injections of testosterone adsorbed on aluminum phosphate. Dosage varied from 50 to 150 mg. per injection. Duration of effect was slightly shorter than that following injection of the macro-crystalline suspension of testosterone propionate. Three of the five patients had reactions at the site of injection. One of these patients experienced a fever lasting for one day. For this reason further clinical trial with this material was abandoned.

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# NOTES AND COMMENTS

## THE EFFECT OF INTRA-OCULAR GRAFTS OF ANTERIOR PITUITARY ON THE THYROID GLAND OF HYPOPHYSECTOMIZED GUINEA PIGS

The extent to which the secretion of thyrotrophic hormone is dependent upon intact neural and vascular connections between the anterior pituitary and the brain has been studied by a variety of techniques with conflicting results. Section of the infundibular stalk in animals kept at normal temperature does not interfere with maintenance of the thyroid according to Mahoney and Sheehan (1936), Brooks (1938), and Uotila (1939), but more recent studies by Westman, Jacobsohn and Okkels (1942) and Brolin (1945) report decreased thyroid function. There is, however, agreement that the increase in thyroid function which normally follows exposure to cold is lacking in the stalk-sectioned animal (Uotila, 1939; Dempsey and Searles, 1943 and Brolin 1946). Ellis and Wiermsa (1945) found thyroid stimulation following electronarcosis, but Green and Harris (1948) obtained no increase in oxygen consumption after electrical stimulation of either the hypophysis or the hypothalamus. The effect of pituitary grafts on thyroid function has been reported only incidental to other studies. Schweizer, Charipper and Haterius (1937) found maintenance of thyroid function whereas Martins (1936) and Cutuly (1941) obtained negative results. In view of the controversial nature of the results reported using anterior pituitary grafts this question has been re-examined using quantitative criteria for thyroid function. Adult male and female guinea pigs were given intra-ocular implants of anterior pituitary tissue, hypophysectomized one to four weeks after implantation and sacrificed at intervals of two to ten weeks after hypophysectomy. Hypophysectomized controls were sacrificed two, three, four and six weeks after operation. The details of these experimental procedures have been reported elsewhere. The thyroid glands were fixed in formol-Zenker, sectioned serially through the middle of the gland at 6u and stained with hematoxylin and triosin. Measurements of acinar cell height as a criterion for thyrotrophic hormone activity (Rawson and Starr 1938) and of follicular diameter as a criterion for colloid storage (Klein 1935) were made on 200 follicles of each gland. The results are summarized in Table I.

### NORMAL AND HYPOPHYSECTOMIZED CONTROLS

*Thyroid weight:* Rowlands (1935) reported no change in thyroid weight following hypophysectomy in the guinea pig. In our experiments there was a gradual increase in both the absolute and relative thyroid weight beginning by the second week after hypophysectomy, reaching a peak by the third week and gradually subsiding until by the sixth week the difference in thyroid weight between the hypophysectomized animals and the normal controls was so slight as to be of questionable significance statistically.

*Colloid storage:* In an attempt to determine to what extent this weight increase was due to storage of colloid the mean follicular diameter was estimated. The mean diameter increased slightly in hypophysectomized animals but the change was barely significant. A more striking difference was observed in the per cent of small (less than 30$\mu$) and large (greater than 60$\mu$) follicles in the population. The number of small follicles decreased sharply to almost 60 per cent of normal by the second week after hypophysectomy and this difference persisted through the sixth week. The number of large follicles increased about 60 per cent by the second week and 100 per cent by the third week and this increase

Received for publication July 31, 1950.

was maintained through the sixth week after operation. In the normal animal the large follicles were present near the center of the gland and the smaller follicles were more peripherally situated. This distribution is the reverse of that found in the mouse and rat. (Dempsey 1949). In the hypophysectomized animal the large follicles were found in the peripheral as well as in the central areas.

TABLE I

| Experimental procedure | No. of animals F = female M = male | Body weight, gm. (range) | Absolute thyroid weight, mg. ± S.E. (range) | Relative thyroid weight, mg./100 gm. ± S.E. (range) | Mean acinar cell height μ ± S.E. (range) | Mean follicle diameter μ ± S.E. (range) | % small follicles (<30μ) ± S.E. (range) | % large follicles (>60μ) ± S.E. (range) |
|---|---|---|---|---|---|---|---|---|
| Normal | 10<br>6F 4M | 467<br>(345–547) | 55.1<br>±3.96<br>(39–78) | 11.8<br>±0.52<br>(9.3–14.1) | 6.5<br>±0.08<br>(6.2–6.9) | 49.1<br>±1.45<br>(42–56) | 20.6<br>±2.4<br>(11–34) | 22.3<br>±2.7<br>(12–36) |
| Hypx 2 Weeks | 4<br>3F 1M | 550[1]<br>(500–590) | 74.5<br>±4.92<br>(61–82) | 13.5<br>±0.70<br>(11.5–14.8) | 5.7<br>±0.04<br>(5.6–5.7) | 53.6<br>±2.59<br>(49–60) | 8.5<br>±1.7<br>(6–13) | 36.8<br>±5.5<br>(25–51) |
| Hypx 3 Weeks | 3<br>1F 2M | 573[1]<br>(475–635) | 111.0<br>±4.99<br>(102–119) | 19.3<br>±0.71<br>(18.3–21.4) | 5.7<br>±0.04<br>(5.6–5.7) | 56.7<br>±4.72<br>(48–65) | 8.5<br>±1.2<br>(6–10) | 47.1<br>±10.7<br>(27–62) |
| Hypx 4 Weeks | 4<br>2F 2M | 531[1]<br>(512–590) | 85.0<br>±7.06<br>(71–104) | 16.0<br>±0.60<br>(14.8–17.6) | 5.4<br>±0.09<br>(5.2–5.6) | 58.6<br>±2.76<br>(52–65) | 6.8<br>±1.9<br>(2–11) | 44.5<br>±5.5<br>(28–49) |
| Hypx 6 Weeks | 5<br>3F 2M | 500[1]<br>(470–550) | 74.2<br>±8.25<br>(54–91) | 14.8<br>±1.72<br>(9.8–17.8) | 5.0<br>±0.07<br>(4.9–5.2) | 57.2<br>±1.99<br>(53–64) | 8.8<br>±2.1<br>(5–14) | 44.6<br>±4.2<br>(36–40) |
| Implanted 5 Weeks. Hypx 3 Weeks | 67F[2]<br>66F[2] | 525[1]<br>550[1] | 91<br>73 | 17.3<br>13.3 | 5.3<br>5.5 | 54<br>58 | 7<br>5 | 44<br>45 |
| Implanted 6 Weeks. Hypx 4 Weeks | 65F[2]<br>62F[2]<br>21M[2]<br>63F[2] | 470[1]<br>525[1]<br>490[1]<br>550[1] | 75<br>96<br>101<br>75 | 15.9<br>18.3<br>20.6<br>13.6 | 4.9<br>6.0<br>5.7<br>5.2 | 53<br>48<br>58<br>53 | 10<br>10<br>5<br>10 | 30<br>26<br>46<br>35 |
| Implanted 7 Weeks. Hypx 6 Weeks | 12F[2] | 620[1] | 80 | 12.9 | 5.5 | 59 | 6 | 46 |
| Implanted 13 Weeks. Hypx 10 Weeks | 3F[2] | 655[1] | 87 | 13.3 | 5.2 | 52 | 16 | 34 |
| Implanted 14 Weeks. Hypx 10½ Weeks | 1F[2] | 580[1] | 78 | 13.4 | 5.0 | 53 | 13 | 39 |
| Normal. Starved. Weight loss 35% | 2M | 498[2]<br>(495–502) | 43.0<br>(42–44) | 8.6<br>(8.5–8.7) | 5.4<br>(5.2–5.6) | 47.0<br>(43–51) | 15.0<br>(9–21) | 23<br>(17–29) |
| Hypx 3 Weeks. Starved. Weight loss 35% | 2M | 530[2] | 58<br>(53–63) | 10.9<br>(10.0–11.9) | 4.9 | 53.0<br>(51–55) | 10.0<br>(9–11) | 37.0<br>(30–44) |
| Implanted 4 Weeks.<br>Hypx 3 Weeks. Starved. Weight loss 39 and 34% | 49F[2]<br>50F[2] | 520[2]<br>480[2] | 49<br>53 | 9.4<br>11.0 | 4.5<br>4.3 | 45<br>52 | 16<br>9 | 18<br>37 |

[1] Weight at hypophysectomy.
[2] Weight before starvation.
[3] Animal number.

Since the thyroid weight passed through a maximum at the third week after hypophysectomy and then decreased, whereas the shift in follicle size which also reached a maximum in the third week persisted through the sixth week, the decrease in thyroid weight after the third week was presumably due, at least in part, to a decrease in the size of the acinar cells.

*Acinar cell height:* The thyroid of the normal guinea pig is known to be inactive com-

pared with that of other animals, and the decrease in cell height after hypophysectomy is correspondingly slight, but statistically significant. The cell height had decreased about 12 per cent during the second and third week, 17 per cent by the fourth week and 23 per cent by the sixth week after hypophysectomy. As described by Rowlands (1935), the acinar cells changed from predominantly low cuboidal in the normal to a lower, almost atrophic, epithelium in the 6 week hypophysectomized animal.

### IMPLANTED-HYPOPHYSECTOMIZED EXPERIMENTAL ANIMALS

The best maintained experimental animal by all criteria used was number 62 in which the acinar cell height was significantly above that of its 4 week hypophysectomized control. The percentage of large follicles approximated that in the normal animal. but the thyroid weight was somewhat above normal. The histological picture approached that of the normal animal. Number 12 had an acinar cell height which was above that of its 6 week hypophysectomized control, though far below normal; neither the distribution of the sizes of the follicles nor the weight of the thyroid gave evidence of normal activity. Number 21 was a borderline case. It gave slight evidence of stimulation in that by all criteria it was more similar to the 3 week hypophysectomized animal than to its 4 week hypophysectomized control. None of the other experimental animals had thyroid glands which were stimulated beyond the levels of the hypophysectomized controls.

In the previous study (Schweizer and Long 1950) of these same animals, the adrenal glands of numbers 67, 62, 21 and 12 were partially maintained; of these the best maintenance was also in numbers 62 and 12.

### STARVATION

In a study of the thyroid gland of underfed guinea pigs which lost 20–30 per cent of body weight in 9–11 days, Rabinovitch (1929) and Stephens (1940) reported changes approaching the effects of hypophysectomy. We have found that with complete withdrawal of food but water ad lib, the guinea pig loses about 35 per cent body weight in 7 days. The acinar cell height in these animals is comparable with that in a guinea pig hypophysectomized 4 weeks but the thyroid weight, unlike that in the hypophysectomized animal, is slightly below normal and the distribution of follicle sizes is similar to that in the normal animal. Animals hypophysectomized 3 weeks and starved to 35 per cent weight loss showed a reduction in acinar cell height 14 per cent below that of the 3 week hypophysectomized control, a reduction of absolute thyroid weight 47 per cent and of relative weight 43 per cent below the hypophysectomized controls. The distribution of follicle sizes was not significantly different from that of the control. These results indicate that some of the effects of inanition are produced directly on the thyroid and are not alone due to reduction in levels of thyrotrophic hormone.

Those animals which were implanted, hypophysectomized and starved (numbers 49 and 50) had acinar cell heights 10 per cent less than their starved hypophysectomized controls and 22 per cent below that of the 3 week hypophysectomized animal, in fact, they had the lowest cell height of any animals studied. The study of the adrenals glands of these animals reported previously (Schweizer and Long 9150) indicated that the grafts were functioning, though at reduced levels. We have no explanation for the greatly reduced cell height of these animals.

### SUMMARY AND CONCLUSIONS

Intraocular grafts of anterior pituitary tissue in the hypophysectomized guinea pig which in a previous study had been shown to give partial maintenance of the adrenal were studied for thyrotrophic activity. Of six animals in which the adrenal cortex was

partially maintained three gave some evidence of thyrotrophic activity, but in all but one, this was at a greatly reduced level. In view of the low thyrotrophic potency of the guinea pig pituitary, it is to be expected that larger grafts would be necessary to maintain thyroid than adrenal function.

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## ROLE OF THE ADRENALS IN THE PLASMA FIBRINOGEN RESPONSE TO TRAUMA

It is well known that the blood fibrinogen is highly increased after trauma (Chanutin, Hortenstine, Cole and Ludewig, 1938). Henriques, Henriques and Selye (1950) observed that this response to trauma is significantly smaller in the adrenalectomized rat.

It seems obvious that in the normal rat the fibrinogen response to trauma could depend on the presence of the adrenal cortex, the adrenal medulla or both. The first possibility is suggested by the knc wn fact that cortical hormones influence protein metabolism (Forsham, Thorn, Bergner and Emerson, 1946; Ingle and Prestrud, 1948; Engel, Schiller and Pentz, 1949) while the role of the adrenal medulla is indicated by the fact that epinephrine injections cause an increase in fibrinogen (Henriques, Henriques and Mattos, 1950).

We therefore decided to study the effect of trauma upon blood fibrinogen in adrenal demedullated rats.

### MATERIAL AND METHODS

Four groups of male Wistar rats, weighing 100 to 150 gm. were used in this experiment. In group I adrenal enucleation was performed, group II was totally adrenalecto-

Received for publication July 19, 1950.

mized, III was sham-operated and IV was left intact. All the animals received 1% sodium chloride instead of drinking water. Thirty days after the operation, trauma (unilateral nephrectomy) was imposed on all the animals. Twenty-four hours later they were bled and killed. Blood samples were taken by puncture of the aorta under ether anesthesia. Blood coagulation was prevented by the use of syringes and needles coated with silicone (Dri-Film 9987, G.E.), the samples being collected in tubes containing 3 mg. of potassium oxalate per ml. of blood.

At autopsy all of the animals were carefully inspected for any detectable infection. The adrenalectomized animals were checked for adrenal remnants. The adrenals of the demedullated rats were removed and fixed in Bouin's fluid for histological examination. Microscopical examinations made at three different levels showed the absence of adrenal medulla in this group.

The plasma fibrinogen was determined by the Cullen and Van Slyke (1920) method with micro Kjeldahl. The digestive mixture used was that of Campbell and Hanna (1937).

## RESULTS

Table 1 summarizes the results. It is to be noted that the adrenal demedullated rats (group I) responded to trauma with the same fibrinogen rise as the sham-operated (group III) or intact controls (group IV) while the adrenalectomized rats (group II) had a significantly lower response ($P \simeq 0.01$).

TABLE 1. INFLUENCE OF ADRENAL ENUCLEATION AND ADRENALECTOMY ON THE FIBRINOGEN INCREASE PRODUCED BY TRAUMA IN THE RAT

| Groups | Procedure | No. of rats | Plasma fibrinogen, mg./100 ml. | Body weights | |
|---|---|---|---|---|---|
| | | | | Initial | Final |
| I | Adrenal-enucleation, 30 days rest, trauma‡ | 9 | 456 ± 23† | 128 (105–160) | 180* (160–205) |
| II | Adrenalectomy, 30 days rest, trauma | 6 | 347 ± 23 | 124 (105–140) | 142 (130–160) |
| III | Sham-operation, 30 days rest, trauma | 6 | 476 ± 27 | 128 (110–150) | 173 (140–220) |
| IV | Normal, 30 days rest, trauma | 9 | 450 ± 15 | 125 (105–160) | 184 (160–230) |

* Before trauma.
† Standard errors.
‡ Unilateral nephrectomy.

## DISCUSSION

The plasma fibrinogen of demedullated rats exposed to trauma as compared to the other groups show indirectly that the adrenal cortical hormones are indispensable for normal fibrinogen synthesis. It appears also that the increase of blood fibrinogen after trauma, at least under the conditions studied, is independent of the adrenal medulla.

The protein catabolic action of the adrenal cortical hormones has been amply substantiated by many different investigators (Long, Katzin and Fry, 1940; Forsham *et al.* 1946; Ingle and Prestrud, 1948; Engel *et al.* 1949). The data presented in this paper suggest on the other hand the importance of the corticoids on protein synthesis.

It can of course be objected that the diminished fibrinogen production in the adrenalectomized animal is due to the unspecific effect of the general condition of the adrenal-

ectomized rat. However this possibility seems unlikely since Henriques, Henriques and Selye (1950) showed that periods of fasting of 72 hours did not affect the fibrinogen increase produced by trauma. Nevertheless further work is needed to clarify this point.

SUMMARY

Experiments are reported on the influence of the adrenal gland on the increase of blood fibrinogen produced by trauma in the rat.

An increase in blood fibrinogen after trauma was observed in adrenal demedullated, sham-operated and normal rats. On the other hand, adrenalectomy reduced significantly this fibrinogen response.

It is suggested that the adrenal cortex plays a rôle in the synthesis of fibrinogen.

*Acknowledgments*

We are indebted to Miss Vera Mondin for preparation of the histological sections.

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## GLUCOSE UTILIZATION *in vitro* BY NORMAL ADULT, IMMATURE AND CRYPTORCHID RAT TESTIS[1]

In a recent study of the metabolism of rat testis *in vitro* (Tepperman, Tepperman and Dick, 1949) it was noted that surviving adult testis exhibits a marked dependence on added glucose for the maintenance of its oxygen consumption whereas cryptorchid testis exhibits a brisk oxygen consumption in the absence of added glucose and a slightly diminished one in its presence. Further, both adult and immature testis showed Respiratory Quotients (R.Q.) between 0.9 and 1.0 in the presence of added glucose, while cryptorchid tissue, under comparable circumstances, yielded an R.Q. of about 0.5. The data to be reported here are to be regarded as an extension of those summarized above. The following experiment demonstrates that both adult and immature testis readily abstract glucose from the medium and oxidize it. Fragments of cryptorchid glands, however, do not utilize added glucose beyond that which can be accounted for by glycolysis.

Received for publication July 19, 1950.

[1] Aided by grants from the American Cancer Society (Administered by the Committee on Growth of the National Research Council) and from the Hendricks Research Fund of the New York State Medical School at Syracuse.

## MATERIAL AND METHODS

Thirty albino rats (Albino Farms) were used in this study. They were maintained with free access to water and Purina Dog Chow. Seven of the animals were rendered bilaterally cryptorchid 22–28 days before sacrifice by means of single, silk sutures which united the capsule of each gland posteriorly with its homolateral abdominal wall.

Oxygen consumption ($Q_{O_2}$) was measured according to the direct method in a Krebs-Henseleit (1932) phosphate buffer of pH 7.4. The flask volumes were approximately 14.5 ml. capacity; from 80–100 mg. of wet tissue was suspended in 2.8 ml. of phosphate buffer which contained 0.2 per cent glucose. The center well contained 0.2 ml. of 20 per cent KOH. $Q_{O_2}$ is expressed as a function of representative dry weight rather than final dry weight.

At the end of two hours of incubation at 37.5°C at a shaking speed of 120 oscillations per minute lactic acid analyses were performed on aliquots of vessel contents according to the technique of Barker and Summerson (1941). Lactic acid formation is expressed conventionally as $Q_G{}^{O_2}$. At the same time other aliquots were taken for glucose analyses for which Nelson's (1944) modification of Somogyi's method was used.

## RESULTS

The experimental results are presented in Table 1. The values observed for $Q^{O_2}$ and $Q_G{}^{O_2}$ closely approximate those reported previously (Tepperman, Tepperman and Dick, 1949). When the quantity of lactic acid formed was subtracted from the amount

TABLE 1. GLUCOSE OXIDATION BY ADULT, CRYPTORCHID AND IMMATURE RAT TESTIS *in vitro*

| Group | Body weight, gms. | Testis weight, mgs./100 gms. of rat | No. of rats | No. of vessels | $Q_{O_2}$ | $Q_G{}^{O_2}$ | Glucose oxidation* (G.O.) |
|---|---|---|---|---|---|---|---|
| Normal adult | 239 | 1.077 | 3 | 14 | 6.4±0.07 | 3.96±0.08 | −10.3±0.8 |
| Adult Cryptorchid | 219 | 0.478 | 7 | 17 | 5.4±0.09 | 3.74±0.17 | +1.8±1.0 |
| Immature | 37 | 0.560 | 20 | 20 | 6.5±0.13 | 6.0 ±0.19 | − 9.0±1.6 |

* Micrograms of glucose disappeared minus lactic acid formed per mg. dry wt. per hr.

of glucose which disappeared from the buffer (G.O.), it was found that the normal adult tissue and the immature tissue yielded values of $-10.3 \pm$ S.E. 0.8 (micrograms per mg. dry wt. per hr.) and $-9.0 \pm$ S. E. 1.6, respectively. The cryptorchid glands, however, gave a value of $+1.8 \pm 1.0$. If one makes the assumption that all of the observed $Q_{O_2}$ was concerned with the oxidation of carbohydrate the theoretical values for G.O. are $-8.5$ for adult tissue, $-8.7$ for the immature, and $-7.2$ for the cryptorchid. In spite of the high standard errors of these groups of data the mean G.O. for the cryptorchid gland is significantly different from that of either of the other tissues at a probability level of far less than 0.01, according to Fisher's "t" test.

## CONCLUSIONS

Surviving cryptorchid testis fails to oxidize glucose added to its incubation medium while both adult testis and immature testis do so at approximately the theoretical rate. These findings are consistent with the facts that cryptorchid testis, in contrast with normal gland, shows a comparatively low R.Q. and exhibits a well-maintained oxygen consumption in the absence of added substrate. It is suggested that the non-germinal

moiety of the gland meets the major part of its energy requirement by the oxidation of non-carbohydrate substrates.

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## ADRENAL CORTICAL RESPONSE OF PROPYLTHIOURACIL TREATED RATS TO THE ADMINISTRATION OF EPINEPHRINE AND ADRENOCORTICOTROPIN*

Zarrow and Money (1949) studied the effect of thiouracil on the adrenal cortex. They concluded that a true atrophy of the adrenal cortex results and that this decrease in cortical size is not due solely to the loss of body weight induced by feeding the goitrogen. In accord with this observation Deane and Greep (1947) in previous histochemical studies of the adrenal cortex following the administration of thiouracil had found that the steroid content of the zona fasciculata was markedly decreased. The former observers then studied the resistance of thiouracil treated animals to cold and found that they were unable to withstand this type of stress as well as do normal animals. A similar conclusion was reached earlier by Ershoff (1948).

The present report is a study of the response of rats treated with propylthiouracil to epinephrine and to adrenocorticotropin. This was done in order to learn whether the failure of thiouracil treated rats to survive a cold stress might indicate a failure to elaborate adrenocorticotropin or failure of the adrenal cortex to respond to stimulation with this fraction.

### METHODS

Mature male rats of the Wistar strain (Carworth Farms) weighing somewhat over 200 grams were employed. They were divided into groups of 12 animals, one group being fed a normal diet and the other groups being placed on a goitrogenic diet for periods of 10, 23, and 43 days. The first group was fed on powdered purina laboratory chow. The propylthiouracil groups were fed on the same diet to which 0.15 per cent by weight of the goitrogen had been added. Free access to tap water was permitted all animals. In each group, four animals were employed as a control for the group, four animals were given 0.5 cc. 1:1000 aqueous epinephrine subcutaneously, and four animals were given 5 mgm. adrenocorticotropin subcutaneously. Four hours following the injections, the animals were killed by the intraperitoneal injection of nembutal. The thyroids and adrenals were dissected clean and weighed on a torsion balance. The ascorbic acid content of the adrenals was determined according to the method of Roe and Kuether.

Received for publication July 7, 1950.
* Aided by a grant from the United States Public Health Service.

TABLE I

| | Initial body weight, grams | Final body weight, grams | Thyroid weight, mgms. | Adrenal weight, mgms. | Adrenal wt. mgms. per 100 grams body weight | Control rats adrenal ascorbic acid concentration mgms. % | ACTH treated rats adrenal ascorbic acid ccncentration mgms. % | ACTH treated rats, per cent fall in adrenal ascorbic acid concentration | Epinephrine treated rats adrenal ascorbic acid concentration mgm. % | Epinephrine treated rats, per cent fall in adrenal ascorbic acid concentration |
|---|---|---|---|---|---|---|---|---|---|---|
| Regular Diet | 236±5.5 | 272±9.2 | 15.2±0.7 | 37.6±2.2 | 13.2±1.0 | 474± 8.1 | 282±24.2 | 41 | 153±27.5 | 68 |
| Propylthiouracil diet for 10 days | 232±3.8 | 226±3.4 | 36.2±2.1 | 28.8±0.9 | 12.7±0.4 | 421±18 | 276±18.1 | 34 | 225±12.3 | 47 |
| Propylthiouracil diet for 23 days | 235±4.9 | 221±4.9 | 50.9±9.1 | 27.3±1.5 | 12.5±0.6 | 367±19.5 | 298±27.6 | 19 | 193±14.8 | 48 |
| Propylthiouracil diet for 43 days | 215±7.5 | 206±8.8 | 67.4±4.2 | 23.8± .8 | 11.7±0.4 | 377±20.1 | 240±26.6 | 26 | 182±17.5 | 52 |

$$\text{S.E.} = \sqrt{\frac{\Sigma d^2}{(n)(n-1)}}$$

RESULTS

Animals given a diet containing propylthiouracil failed to gain weight in contrast to the increase in weight noted in the controls. The increase in thyroid weight and the decrease in adrenal weight was greater the longer the animals were kept on the goitrogen. When the adrenal weight per 100 grams of body weight is calculated in order to correct for the wide difference in mean final weights of the various groups, the values for the ratio indicate that the degree of atrophy of the cortex depends on the length of time during which the goitrogen was administered. However, although the trend is evident, the difference at the end of 43 days is not sufficient to satisfy rigid statistical criteria of involution.

The content of ascorbic acid of the adrenal gland is reduced following treatment with the goitrogen. In all groups receiving a goitrogenic diet, however, in spite of marked thyroidal hypertrophy and apparent adrenal involution, the response to the administration of epinephrine and of adrenocorticotropin is similar to that observed in the animals on the non-goitrogenic diet.

DISCUSSION

Ershoff (1948) observed that mature rats placed on a goitrogenic diet for four weeks were unable to resist cold stress. Zarrow and Money (1949) found similar results in mature rats kept on such a diet for 10 weeks. They did not subject any mature group treated for less than this period of time to stress. In young rats, however, an inadequate response to cold was noted after only three weeks of therapy. Numerous investigators have demonstrated that the adrenal cortex will protect animals against cold, and this has formed the basis for one method of biological assay of cortical extract. Zarrow and Money (1949) observed that atrophy of the adrenal cortex may be noted after only two weeks of goitrogen administration and that the adrenal weight failed to increase in thiouracil treated rats exposed to cold as it did in normal control animals. In view of these findings and the reported depletion of ketosteroids histochemically following goitrogen administration, these observers postulated that the lack of resistance was either due to failure of the elaboration of adrenocorticotropin or an inability of the adrenal cortex to respond to this hypophyseal fraction. However, Ershoff (1948) demonstrated that the thiouracil treated animals could be protected from death due to cold by thyrotxin but not by adrenal cortical extract.

Our results would indicate that although the ingestion of a goitrogen will result in involution of the adrenal cortex and a decrease in its ascorbic acid content, the cortex will nevertheless respond to the injection of adrenocorticotropin or to epinephrine. In other unpublished experiments we found that the degree of hypertrophy of the adrenal cortex induced by the administration of adrenocorticotropin over a period of ten days was identical in both the normal and the propylthiouracil treated animal.

Although resistance to cold may be employed as a test of adrenal function in the euthyroid animal, this is not necessarily true in the hypothyroid animal. Animals adapted to a cold environment prior to adrenalectomy survive a cold stress indefinitely (LeBlond and Gross, 1943; Selye, 1937). In contrast, previous exposure to cold only slightly increased the survival time of subsequently thyroidectomized animals subject to this stress (LeBlond and Gross, 1943). It is thus apparent that cold is not the proper type of stress with which to test adrenal cortical function in the hypothyroid animal. The thyroid of an animal subjected to cold increases its secretion of thyroid hormone (Dempsey and Astwood, 1943). It is possible that the adrenal hypertrophy noted in animals exposed to a cold environment may in part be secondary to the increased secretion of thyroid hor-

mone since the administration of this fraction is known to produce adrenal cortical hypertrophy.

SUMMARY

Rats treated with goitrogen for periods of up to 43 days react normally to the administration of adrenocorticotropin and epinephrine as demonstrated by the adrenal content of ascorbic acid in spite of involution of the cortex and a decreased resting content of Vitamin C.

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